

Different somatic alterations of the *HRPT2* gene in a patient with recurrent sporadic primary hyperparathyroidism carrying an *HRPT2* germline mutation

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Abstract

Early onset of primary hyperparathyroidism (PHPT) and multiglandular involvement suggest a familial form in which germline mutation of a *PHPT*-related gene(s) and a somatic event at the same locus can be often demonstrated. We investigated the involvement of multiple endocrine neoplasia type 1 (*MEN1*) and *HRPT2* genes in a 39-year-old man with recurrent PHPT. PHPT was firstly diagnosed at the age of 21 and the patient had two recurrences separated by extended periods of normocalcemia. This unusual history prompted us to investigate other family members and study the *MEN1* and *HRPT2* genes. An *HRPT2* germline missense mutation in exon 3 (R91P) was found in the index case, which was associated with different *HRPT2* somatic alterations in each of the three examined parathyroid tumors. These findings are consistent with Knudson's 'two hit' concept of biallelic inactivation of classical tumor suppressor genes. Screening of 15 asymptomatic relatives was negative for the R91P germline mutation. All the three abnormal parathyroid specimens showed cystic features at histology and were negative for parafibromin immunostaining. In one specimen, diffuse parafibromin staining was evident in a rim of normal parathyroid tissue surrounding the adenomatous lesion. Our study shows that different somatic genetic events at the *HRPT2* locus are responsible for the asynchronous occurrence of multiple adenomas in a patient carrying an *HRPT2* germline mutation. The finding of diffuse parafibromin staining in a rim of normal parathyroid tissue, but not in the contiguous adenomatous lesion, reinforces the concept that loss of parafibromin expression is responsible for the development of parathyroid tumors in this setting.

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Introduction

Primary hyperparathyroidism (PHPT) is one of the most common endocrine disorders, especially in postmenopausal women in whom it reaches a prevalence of 2–3% (Adami *et al.* 2002). A single, benign parathyroid adenoma is responsible for sporadic PHPT in 80–85% of cases, the remaining being caused by multiple adenomas, hyperplasia of all parathyroid glands, and rarely by carcinoma (Marx 2000). On the other hand, multiglandular involvement is typically found in familial forms of PHPT, namely multiple endocrine neoplasia

types 1 and 2A (MEN 1 and MEN 2A), hyperparathyroidism–jaw tumor syndrome (HPT–JT), or familial isolated hyperparathyroidism (FIHP) (Marx *et al.* 2002). These inherited forms of PHPT typically present at an earlier age than sporadic forms of PHPT and occur with equal frequencies in both sexes. Thus, the finding of multiglandular involvement at surgery or recurrence of PHPT in the same individual, particularly in a young subject, could suggest a hereditary syndrome.

The identification of genes responsible for familial PHPT (*MEN1*, *HRPT2*, *CASR*, and *RET* genes) has

increased our knowledge about parathyroid tumorigenesis (Chandrasekharappa et al. 1997, Carpten et al. 2002, Marx et al. 2002). *MEN1* and *HRPT2* function as tumor suppressor genes, consistent with the Knudson 'two hit' hypothesis. The second hit often causes loss of heterozygosity (LOH) of a large chromosomal region, as in the majority (~70–90%) of *MEN1*-associated tumors (Larsson et al. 1988, Friedman et al. 1989, Thakker et al. 1989, Pannett & Thakker 2001), but other mechanisms such as smaller deletions and point mutations that may also inactivate the gene, can occur and seem to be preferentially involved in *HRPT2*-related tumors (~30–50%; Arnold et al. 2002, Carpten et al. 2002, Howell et al. 2003, Shattuck et al. 2003, Cetani et al. 2004, Villablanca et al. 2004, Moon et al. 2005, Bradley et al. 2006, Kelly et al. 2006, Mizusawa et al. 2006). Hereditary syndrome when compared with sporadic parathyroid tumors shows early onset age and multiplicity, because each parathyroid cell has 'one hit' by inheritance. Thus, subtotal or total parathyroidectomy is usually performed for the cure of familial PHPT, whereas the excision of the single diseased gland is sufficient in most cases of sporadic PHPT.

In this report, we describe a 39-year-old man with sporadic PHPT, who had two recurrences after successful surgery over a 17-year follow-up. This unusual history prompted us to perform genetic studies, which led to the identification of a *HRPT2* germline mutation associated with independent *HRPT2* somatic alterations (mutations or LOH at the same locus) in the different parathyroid tumors.

Materials and methods

Case report

The proband, a 39-year-old man, referred to our Department in February 1998 for recurrent sporadic PHPT. In 1987, at the age of 21 years, a severe form of PHPT was diagnosed (serum calcium 17.3 mg/dl (normal range 8.2–10.2); C-PTH 3.17 ng/ml (< 0.88 ng/ml), and *osteitis fibrosa cystica*). On surgery, a 3-cm right inferior (RI) parathyroid gland was removed. Three additional parathyroid glands were identified and showed a normal appearance; a biopsy of the left superior (LS) parathyroid was carried out. Histological examination showed a RI parathyroid adenoma and normal parathyroid tissue of the LS gland. Three years later, in 1990, recurrence of PHPT was documented but no treatment was advised. In 1993, the patient underwent cervical exploration and a 1.5 cm right superior (RS) parathyroid gland was

excised and both left glands appeared grossly normal. Histology revealed an oxyphilic adenoma. Serum calcium and PTH remained normal until 1997 when a further recurrence of PHPT was documented and the patient referred to our department. The patient was in good health. Serum calcium and PTH were mildly elevated (10.6 mg/dl and 72 pg/ml (normal range 10–65) respectively). There was no evidence of *MEN1*-associated neoplasia. No kidney lesions or jaw tumor were detected. Parathyroidectomy was advised, but the patient was lost to follow-up until 2004, when he was referred again to our department. The patient was in good general condition. No cutaneous lesions were evident at physical examination. Serum total and ionized calcium (12.2 mg/dl (2.70 mmol/l) and 5.5 mg/dl (1.75 mmol/l) respectively), PTH (260 pg/ml), and markers of bone turnover were elevated. Prolactin (PRL), growth hormone (GH), insulin-like growth factor-I (IGF-I), thyroid-stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), cortisol, follicle-stimulating hormone (FSH), luteinizing hormone (LH), insulin, gastrin, vasoactive intestinal polypeptide (VIP), glucagon, serotonin, adrenalin, noradrenalin and glucose concentrations were within the normal range. Neck imaging studies (ultrasound, 99m-Tc-sestamibi scan and computerized tomography (CT)) showed two enlarged parathyroids in the left side of the neck. No pancreatic or adrenal lesions were found by ultrasound or CT. Chest X-ray and CT were normal. Orthopantography of the jaw was negative. The patient was submitted to surgery with removal of the LS (1.5 cm) and left inferior (LI; 1.0 cm) parathyroid glands. Histological examination showed chief cell adenomas. After a transient hypocalcemia, serum calcium and PTH remained normal until March 2006.

Fifteen first degree relatives were available for further investigations. Serum calcium and PTH were in the normal range in all cases.

Informed consent was obtained from the patient and his relatives for all procedures used in the present study. The study was approved by the local ethical committee.

Methods

Tissue samples

Tissues were obtained at the time of surgery, immediately snap frozen in liquid nitrogen and stored at -80°C until use. Tissue sample of the RI parathyroid gland was not available.

Genetic studies

Genomic DNA was isolated from peripheral blood leukocytes and parathyroid tissues by the standard proteinase K-SDS digestion and the phenol/chloroform method. Allelic deletions and direct sequencing of *HRPT2* and *MEN1* genes were assessed in both blood leukocytes and tumor DNAs as described previously (Cetani *et al.* 2004). Nucleotide sequences were determined on double strands at least twice. The intragenic polymorphisms in introns 10 and 14 of the *HRPT2* gene and the microsatellite markers (PYGM and D11S449) flanking the *MEN1* gene were used. The region of interest of the *HRPT2* gene was also amplified using constitutional DNA of all family members and 55 unrelated controls of Italian origin (110 chromosomes). The sequence abnormality found in the constitutional DNA was confirmed by restriction enzyme analysis.

Immunohistochemistry

Sections of the left parathyroid tumors were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase activity was blocked by incubating the slides in 1% hydrogen peroxide in methanol for 10 min. In order to unmask the antigen, the slides were microwave-treated in 10 mM citrate buffer, pH 6.0 for 10 min. After blocking nonspecific staining with normal serum, the sections were incubated for 1 h with the primary anti-parafibromin monoclonal antibody (Tan *et al.* 2004; kindly donated by Bin Tean Teh), which was used at 1:50 dilution. This antibody is directed against the portion of the protein corresponding to amino acid positions 87–100. The sections were then incubated with biotin-labeled secondary antibody (dilution 1:500) and avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA) for 30 min each. Sites of binding were visualized using 3,3'-diaminobenzidine as chromogen. Finally, sections were counterstained with hematoxylin, dehydrated, and mounted. The positive control was normal parathyroid and two negative controls included experiments omitting primary antibody or using primary antibody pre-absorbed with a 20-fold excess of the immunizing peptide. For each case, six different sections were analyzed.

Tumors were scored as positive if specific nuclear staining was detected and the staining was quantified according to the percentage of positive cells, independently of the intensity of staining (Cetani *et al.* 2007). Tumors were scored as negative when no tumor cells showed a specific nuclear staining.

Results

Genetic studies

All tissue specimens were heterozygous for the *MEN1* gene flanking markers *D11S449* and *PYGM*, thus indicating the absence of large chromosomal deletions. Direct sequencing of the *MEN1* gene did not reveal any mutation.

Results of *HRPT2* gene are summarized in Table 1 and shown in Fig. 1. LOH of both intragenic markers was identified in the LS parathyroid specimens. Retained heterozygosity was present in the other parathyroid samples. Sequence analysis of PCR-amplified tumor and germline DNAs revealed two missense (R91P and A2S) and one nonsense (Y54X) *HRPT2* mutations. The R91P mutation was germline; the A2S mutation was found only in the LI gland, and the Y54X only in the RS. The latter mutation has been previously reported in a parathyroid cancer (Howell *et al.* 2003, Shattuck *et al.* 2003). The two missense mutations alter evolutionarily conserved amino acids (alanine to a serine and arginine to a proline). The substitution of the nonpolar hydrophobic alanine for a polar hydrophilic serine in the A2S mutation and of an arginine for a helix-breaker proline in the R91P mutation may likely lead to deleterious structural alterations of parafibromin that may affect its function.

The R91P germline mutation led to the loss of a *TaqI* site providing a convenient diagnostic test to confirm the presence of mutation in the proband and to demonstrate its absence in the other family members and 55 control DNAs. As indicated in Fig. 1, digestion of a fragment of exon 3 containing the R91P mutation with *TaqI* produced a 163 (mutant allele), 85 and 78 bp (wild-type allele) fragments. All relatives were also screened for the mutation by sequencing. The R91P mutation was absent in the patient's mother and sister and in three paternal first-degree cousins. We could not investigate the father because he was deceased, but the paternal grandfather

Table 1 Genetic studies in parathyroid tissues

Parathyroid gland	<i>HRPT2</i> sequence	LOH <i>HRPT2</i> locus ^a
Right inferior	NA	NA ^b
Right superior	Y54X Somatic R91P Germline	Retention
Left inferior	A2S Somatic R91P Germline	Retention
Left superior	R91P Germline	LOH

^aIntragenic markers in introns 10 and 14 were studied (Shattuck *et al.* 2003).

^bNot available.

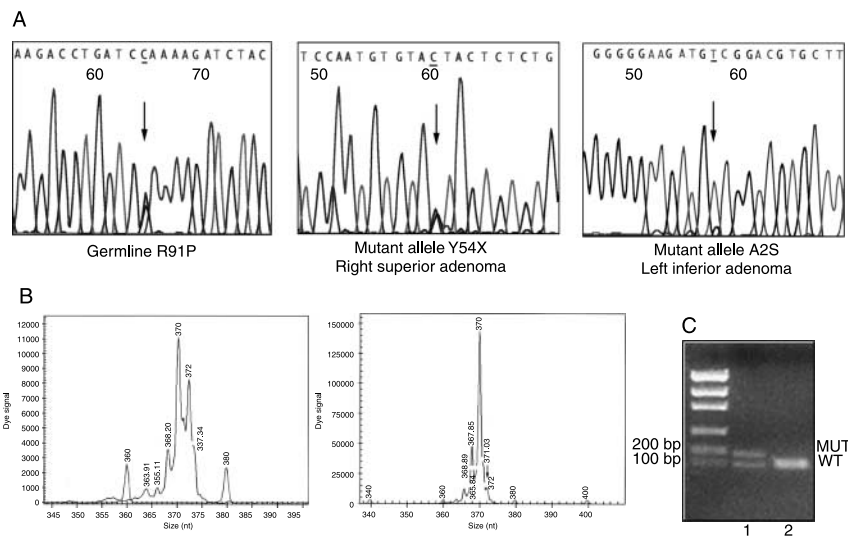


Figure 1 Mutational analysis, LOH, and restriction enzyme analyses of *HRPT2* gene. (A) Sequencing chromatograms. Left panel: heterozygous R91P germline mutation in exon 3 (CGA > CCA); middle panel: heterozygous Y54X somatic mutation detected in the RS adenoma (TAC > TAG); right panel: heterozygous A2S somatic mutation detected in the LI adenoma (GCG > TCG). (B) LOH analysis. Tumor DNA shows a LOH (arrow) using intron 10 polymorphism. (C) Detection of a R91P mutation in exon 3 by restriction endonuclease analysis. The mutation results in the loss of a *TaqI* restriction enzyme site. Following PCR with appropriate primers of a fragment of exon 3 and cleavage with *TaqI*, a band of 163 bp (mutant allele) and two bands of 85 and 78 bp are shown in the patient (lane 1) and only the latter bands in the control subject (lane 2). The positions of the size markers at 100 and 200 bp are indicated.

and all the father's six siblings were negative. These data suggest that the R91P germline mutation occurred *de novo* in our patient. This mutation was not detected in the patient's three sons.

Immunohistochemistry

Normal parathyroid gland was used as the positive control and showed a diffuse nuclear staining in ~90% of chief cells, without staining of adipose and connective tissue or blood vessels. All abnormal parathyroid specimens showed cystic features and were negative for parafibromin expression (Fig. 2). Interestingly, parafibromin staining was retained in a rim of normal parathyroid tissue surrounding the LI adenoma. The percentage of positive cells and the intensity of staining were comparable to those observed in the normal parathyroid gland.

Discussion

The medical history and the apparent success of initial surgery (RI parathyroidectomy) were consistent with the diagnosis of sporadic PHPT due to a single parathyroid adenoma, but the subsequent follow-up has clearly shown that this was not the case. Indeed, the patient had two recurrences of PHPT, which occurred 3 and 4 years after normalization of serum calcium and PTH, in both cases following the excision of a single enlarged

parathyroid gland, with a histological picture of adenoma. Moreover, in both occasions, evidence of histologically normal parathyroid tissue was available. Thus, after the second adenomectomy, which was followed by normocalcemia, it was reasonable to believe that the patient was affected by double parathyroid adenomas. However, as mentioned before, a further relapse of PHPT occurred and, at this time, two enlarged parathyroid glands were removed and chief cell adenomas were found at histology.

The early presentation of PHPT and the evidence of subsequent multiglandular involvement, despite the absence of family history and other associated syndromic manifestations, suggested a possible hereditary form of PHPT. We studied the *MEN1* and *HRPT2* genes, although we would not have had as high an expectation for *MEN1*, since the asynchrony, the 'one adenoma at a time' presentation and the presence of cystic changes in parathyroid tumors suggest the involvement of the *HRPT2* gene rather than the *MEN1* gene. We found a *HRPT2* germline mutation, which was paired with different acquired genetic abnormalities in the three abnormal parathyroid glands we studied. Interestingly, the *HRPT2* germline mutation was missense. Nonsense or frameshift mutations, resulting in truncated proteins, usually occur in the HPT-JT syndrome and parathyroid cancer (Carpten et al. 2002, Howell et al. 2003, Shattuck et al.

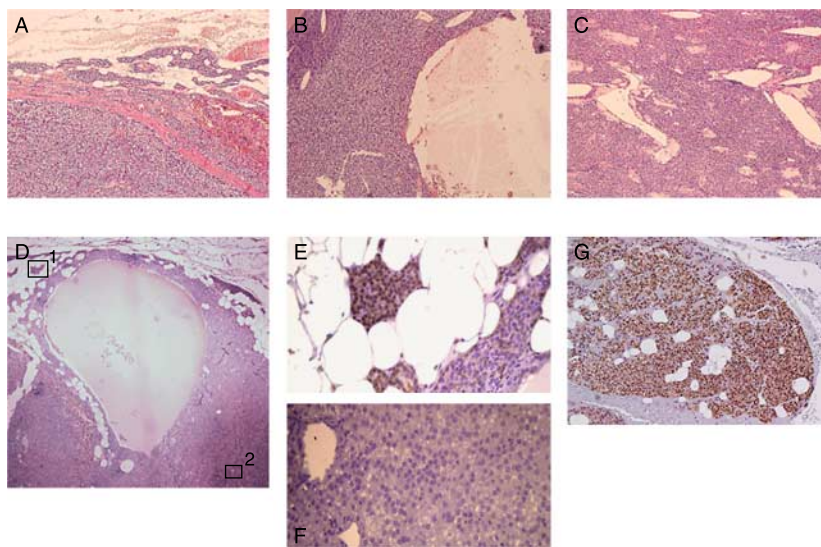


Figure 2 Histology (upper panels) and parafibromin staining (lower panels). (A) The adenomatous tissue and a rim of normal tissue separated by the capsule are shown (LI parathyroid adenoma, hematoxylin-eosin, $\times 100$). (B) Large cystic lesion of LI parathyroid adenoma (hematoxylin-eosin, $\times 100$). (C) Multiple cystic lesions of LS parathyroid adenoma (hematoxylin-eosin, $\times 100$). (D) Low magnification of the LI parathyroid adenoma showing a rim of normal parathyroid tissue (immunohistochemistry, $\times 40$). (E) Magnification of inset 1 of panel D showing a diffuse nuclear immunoreactivity of normal parathyroid tissue surrounding the adenoma (immunohistochemistry, $\times 400$). (F) Magnification of inset 2 of panel D showing absence of nuclear immunoreactivity of adenomatous tissue (immunohistochemistry, $\times 400$). (G) Normal parathyroid gland showing a diffuse nuclear immunoreactivity (immunohistochemistry, $\times 200$).

2003, Cetani *et al.* 2004, Bradley *et al.* 2006). It has been hypothesized that missense mutations, resulting in abnormal proteins, may have a lower phenotypic penetrance. As a matter of fact, an association between *MEN1* gene missense mutations and FIHP has been reported by Pannett & Thakker (2001). A similar *HRPT2* mutation spectrum has been suggested in FIHP versus HPT–JT. Indeed, an overall analysis of the *HRPT2* germline mutations identified by this study together with the 11 previously reported in FIHP (Carpten *et al.* 2002, Howell *et al.* 2003, Cetani *et al.* 2004, Simonds *et al.* 2004, Villablanca *et al.* 2004, Bradley *et al.* 2005a,b, 2006, Guarnieri *et al.* 2006, Kelly *et al.* 2006, Mizusawa *et al.* 2006) reveals that 16.7% are missense mutations as opposed to 9.5% in HPT–JT (Carpten *et al.* 2002, Howell *et al.* 2003, Bradley *et al.* 2005b, 2006, Moon *et al.* 2005, Aldred *et al.* 2006, Mizusawa *et al.* 2006, Yamashita *et al.* 2007). Thus, the presence of a missense mutation might explain the absence of an obvious family history in apparently sporadic PHPT patients. This is in keeping with the findings in our patient, in whom, despite the R91P germinal *HRPT2* mutation, no other phenotypic features of classical HPT–JT were present. On the other hand, this mutation might have increased parathyroid proliferation and therefore the risk of additional somatic events, responsible for the

asynchronous development of parathyroid gland abnormalities in our patient. Indeed, different *HRPT2* gene somatic alterations were found in each individual excised gland. Asynchronous somatic events may account for the usual clinical history of recurrent PHPT in HPT–JT, in which each recurrence occurs as a single gland disease, separated by periods of normocalcemia. The importance of a second somatic hit at the *HRPT2* locus in the pathogenesis of parathyroid adenomas in our patient is strongly supported by the finding of positive parafibromin staining in a small rim of normal tissue surrounding the LI adenoma.

Only one study has investigated genetic alterations in multiple tumors occurring in the same patient with sporadic PHPT (five cases of double adenomas; Dwight *et al.* 2002). The tumors were examined for LOH at distal 1p, HPT–JT locus at 1q-21-32 and flanking regions, and *MEN1* locus at 11q13. Different genetic events were found in paired glands. While LOH was found in one gland, the other gland showed either LOH in another locus or no allelic loss. In particular, three tumors had LOH at 11q13 associated with a somatic *MEN1* mutation in the other allele, but not in the germline DNA.

The *HRPT2* germline mutation found in the present study involves amino acid 91 and, therefore, it is possible that the anti-parafibromin antibody used in the present study, which is directed against amino acids 87–100, may

not recognize the protein encoded by the germline mutated *HRPT2* allele. Thus, the absence of parafibromin staining would not demonstrate its inactivation in the tumor. On the other hand, the replacement of an arginine for a helix-breaker proline suggests parafibromin inactivation.

Our patient had removed four abnormal parathyroid glands, which were asynchronously involved over a 17-year period. The normal serum calcium and PTH after the last surgical procedure suggest the presence of supernumerary parathyroid gland(s). Alternatively, it is possible that the patient has a small amount of locally invasive parathyroid cancer in the right side of the neck, which was not re-explored at the last surgery, or metastatic disease elsewhere. Re-evaluation of all histological sections of right-side tumors by our pathologist (PV) showed no histologic features suggestive of potential malignancy. Since *HRPT2* mutation carriers are at risk for recurrence of PHPT or parathyroid cancer, regular follow-up is mandatory in our patient. Frequent surveillance may allow an early detection and cure of PHPT, before a possible development of malignancy (Guarnieri et al. 2006, Kelly et al. 2006).

In conclusion, our study shows that different somatic genetic events at the *HRPT2* locus are associated with the asynchronous occurrence of multiple adenomas in a patient carrying an *HRPT2* germline mutation. The finding of diffuse parafibromin staining in a rim of normal parathyroid tissue, but not in the adenomatous lesion, further reinforces the concept that loss of parafibromin expression is responsible for the development of such parathyroid tumors.

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