CLINICAL STUDY

MEN1 intragenic deletions may represent the most prevalent somatic event in sporadic primary hyperparathyroidism

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Abstract

Objective: Primary hyperparathyroidism (pHPT) is characterised by an inappropriate over production of parathyroid hormone and it is the most frequent pathological condition of the parathyroid glands. A minority of the cases belong to familial forms, but most of them are sporadic. The genetic alterations underlying the sporadic forms of pHPT remain poorly understood. The main goal of our study is to perform the molecular characterisation of a series of sporadic pHPT cases.

Design and methods: We have studied matched blood and tumour from 24 patients with pHPT, who went to a medical appointment in Hospital Pedro Hispano. Informed consent was obtained from all individuals. The *MEN1*, *RET* and *CDKN1B* molecular study was carried out in the germline DNA by PCR/SSCP and direct sequencing. Parathyroid tumours were further analysed by the same methods for *MEN1*, *CDKN1B* and *CTNNB1* genetic alterations. The multiplex ligation-dependent probe amplification technique enabled the evaluation of *MEN1* gene deletions. Protein expression for menin, cyclin D1, parafibromin, p27^{Kip1}, β -catenin and Ki-67 was conducted by immuno-histochemistry.

Results: The study of parathyroid tumours detected two somatic *MEN1* mutations (c.249_252delGTCT and c.115_163del49bp) and revealed the presence of *MEN1* intragenic deletions in 54% (13/24) of the tumours. In *RET* and *CDKN1B* genes only previously described, non-pathogenic variants were found. Cyclin D1 protein was overexpressed in 13% (3/24) of tumours.

Conclusions: These results suggest that *MEN1* alterations, remarkably intragenic deletions, may represent the most prevalent genetic alteration in sporadic parathyroid tumours.

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Introduction

Primary hyperparathyroidism (pHPT) represents one of the most common endocrine disorders in western populations (1). Clinically, the hallmark of pHPT is characterised by a disproportionate secretion of parathyroid hormone (PTH) that culminates in raised serum calcium causing adverse effects on many organs. Sporadic cases of pHPT are often attributed to the presence of a benign, single adenoma in 80% of the cases, to multiglandular parathyroid hyperplasia in 15–20% of cases and to parathyroid carcinoma in about 1% (2).

Non-familial pHPT accounts for more than 95% of the cases, while the remaining cases occur in the setting of inherited disorders: multiple endocrine neoplasia type 1 or type 2 (MEN1 or MEN2) and more rarely HPT–jaw tumour (HPT–JT) syndrome, caused by germline mutations in *MEN1* tumour suppressor gene, *RET*

proto-oncogene and *HRPT2* (*CDC73*) tumour suppressor gene (3, 4). Additionally, germline mutations in *CDKN1B*, the gene coding for the cyclin-dependent kinase inhibitor p27^{Kip1}, were identified in a few families bearing a MEN1-like syndrome, thus evidencing a p27^{Kip1} contribution in pHPT development (5). Therefore, the identification of *CDKN1B* germline mutations has defined a novel multiple endocrine neoplasia syndrome, MEN4 (OMIM No.610755) (6). This entity clinically closer to pHPT is characterised by parathyroid involvement and, less typically, by pituitary adenomas and other endocrine features (7). Until now, six germline *CDKN1B* mutations have been found in patients with a MEN1-like phenotype but were negative for *MEN1* mutations (8). At variance with the well-known genetic alterations underneath familial pHPT, the molecular alterations underlying the sporadic forms still remain poorly understood. In an attempt to

elucidate the genetic alterations of sporadic pHPT, classical tumour suppressor genes have been investigated (e.g. *TP53*, *RB* and those encoding cyclin-dependent kinase inhibitors – *CDKN2A*, *CDKN2C* and *CDKN2D*), but evidence of somatic inactivation of these genes in parathyroid tumours was rarely found (9, 10).

β -Catenin is a transcription-activating protein with oncogenic potential, and a mutation-dominant effect has been hypothesised; until now, only somatic heterozygous mutations have been identified for *CTNNB1* (sporadic colorectal carcinoma, hepatoblastoma) (11). Deregulated activation of the Wnt signalling pathway, through stabilising β -catenin mutations in exon 3 or inactivating APC mutations, is involved in the majority of colorectal cancer (11, 12). Also in primary (parathyroid tumours) and in secondary HPT (hyperplastic parathyroid glands), excessive β -catenin signalling was reported as a major alteration. Bjorklund *et al.* (13) demonstrated activation of the canonical pathway through excessive β -catenin signalling and the presence of a stabilising mutation in a small subset of cases. Conversely, independent studies of parathyroid adenoma series have failed to identify mutations in the β -catenin gene, *CTNNB1* (14).

To date, two genes have established roles in the development of sporadic parathyroid tumours: *MEN1* and cyclin D1 (*CCND1*), both located at chromosome 11 (11q13). *MEN1* tumour suppressor gene, which was identified in 1997 (15, 16), encompasses ten exons that encode a 610 amino acid protein referred to as menin (17, 18). Using polymorphic markers, loss of *MEN1* locus was observed in 25–40% of sporadic parathyroid tumours, and an accompanying inactivating mutation was detected in about 50% of these tumours (19, 20, 21).

The *CCND1* gene (also known as *PRAD1* – parathyroid adenomatosis 1) encodes a protein product that is a key regulator of the cell cycle. *cCCND1* was first recognised as an oncogene in parathyroid tumours due its rearrangement with the *PTH* gene promoter region (22). Overexpression of this cell cycle regulator is observed in several human cancers such as breast, colon, lymphoma, melanoma and prostate (23). Cyclin D1 overexpression has been implicated through abnormal expression levels in the pathogenesis of 20–40% of sporadic parathyroid tumours (24, 25).

Regarding parathyroid carcinomas, inactivating mutations of parafibromin are the most common genetic events with a mutation rate ranging from 66 to 100% and concomitant loss of immunoreactivity. Consequently, it was advanced that parafibromin protein expression discriminates parathyroid carcinoma from benign parathyroid lesions (26).

The aim of this work is to perform a thorough molecular characterisation of a series of sporadic pHPT cases in order to contribute to a more accurate diagnosis and unveil pivotal steps in the etiopathogenesis.

Materials and methods

Subjects

A retrospective review of a database of patients with pHPT was conducted in Hospital Pedro Hispano (HPH), Portugal, retrieving 80 patients. Upon attainment of informed consent, 30 patients (8 males; 22 females; average age 61 years, ranging from 32 to 89 years) had a medical appointment consisting of a systematic interrogation about their personal, familial history and physical examination. The diagnosis of sporadic pHPT was based on elevated levels of serum calcium (≥ 2.75 mmol/l) and PTH (≥ 6.8 pmol/l) and a negative family history. The study was approved by the ethics committee of HPH, and all the procedures were in accordance with the institutional and national ethical rules.

Genetic analysis

Germline study of *MEN1*, *RET* and *CDKN1B* genes was performed in DNA extracted from peripheral blood leukocytes of 30 patients (27). The study of somatic alterations was carried out in DNA extracted (Puregene kit, Gentra Systems, Inc., Minneapolis, MN, USA) from formalin-fixed paraffin-embedded (FFPE) tissues from 24 patients, provided by the Department of Pathology of HPH (FFPE material from six patients was not available, not representative or degraded). The histological slides of the parathyroid lesions were reviewed by a pathologist (EF), with 19 tumours (79%) classified as adenomas (uniglandular disease) and five (21%) as hyperplastic glands (multiglandular disease).

Mutational analysis

The *MEN1* (ENSG00000133895) gene coding exons 2–10, *RET* (ENSG00000165731) gene coding exons 10, 11 and 13–16 and the entire *CDKN1B* (ENSG00000111276) gene encoding exons 1 and 2 were amplified in the germline and somatic DNA samples by PCR, using specific primers and annealing temperatures (primers and annealing temperatures summarised in Supplementary Data: Tables 1 and 2). The PCR was performed in a volume of 25 μ l containing 5 μ l of 5 $\times$ PCR buffer (Bioron, Ludwigshafen, Germany), 2.5 mM $MgCl_2$ (Bioron), 1 μ l deoxynucleoside triphosphates (5 mmol/l each) (Bioron), 0.24 μ M of each primer, 1U *Taq* DNA Polymerase (Bioron) and 50–100 ng genomic DNA. Thermal cycling conditions used were as follows: an initial denaturation step at 94 °C for 3 min, followed by 35–40 cycles of denaturation at 94 °C for 20 s, annealing at 55–65 °C for 30 s, extension at 72 °C for 45 s and a final extension at 72 °C for 10 min.

The samples were analysed by SSCP followed by direct DNA sequencing in the cases presenting abnormal electrophoresis mobility. SSCP was performed by denaturing samples and running them in a vertical

electrophoresis using a Mutation Detection Enhancement (MDE) gel (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) at 8 or 20 °C (exon dependent). Results were revealed using silver staining. PCR products were subjected to sequencing in both strands using the Big Dye Terminator Cycle Sequencing Reaction kit (Applied Biosystems, Life Technologies Corporation) and analysed on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The *CDKN1B* exons and *CTNNB1* exon 3 analysis was performed by PCR and direct sequencing as described elsewhere (28).

Multiplex ligation-dependent probe amplification

DNA from germline ($n=30$) and tumour ($n=24$) samples was analysed for genetic deletions using multiplex ligation-dependent probe amplification (MLPA) kit (SALSA MLPA KIT PO17-B1 MEN1 MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. MLPA fragments were run on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), and the data were analysed using Coffalyser software (MRC Holland). MLPA results were reproduced at least three times.

Molecular allelotyping

Loss of heterozygosity (LOH) analysis was performed on DNA from tumour and matched peripheral leukocyte DNA. Three microsatellite markers at 11q13 (D11S956, PYGM and RH27780) region were used to evaluate the extension of *MEN1* deletions provided by MLPA. In each primer pair, one nucleotide was labelled with TET or HEX. TAMRA500 (Applied Biosystems) was used as an internal size standard. In order to analyse allelic loss within the *MEN1* locus, a polymorphism in exon 9 (SNP ID: rs2071313) was genotyped.

Fragment size analysis was performed using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The data were analysed regarding allele size and quantitative information on allele height and area. LOH was defined according to the formula: $LOH = (T2 \times N1) / (T1 \times N2)$, where T was the tumour sample, N was the corresponding blood sample and 1 and 2 were the peak areas of smaller and larger alleles. LOH was defined with values of LOH index <0.6 or >1.67 .

Microsatellite instability (MSI) was evaluated using five quasimonomorphic mononucleotide repeats named BAT-26, BAT-25, NR-24, NR-21 and NR-27 as described previously (29).

Immunohistochemistry

Sections of the parathyroid lesions were deparaffinised and rehydrated in alcohol. Endogenous peroxidase activity was quenched by incubating the slides with 3% (v/v) hydrogen peroxide in methanol.

Microwave-treated antigen retrieval was used for cyclin D1, β -catenin, Ki-67 and p27^{Kip1} in 10 mM citrate buffer, pH=6, and EDTA buffer, pH=9, for parafibromin protein. After blocking nonspecific staining (Ultra V Block, Thermo Fisher Scientific, Inc., Ottawa, ON, Canada), primary antibody was applied (cyclin D1 SP4, 1:100 (Neomarkers, Inc., Fremont, CA, USA); β -catenin, 1:4000 (c2206-1ML, Sigma-Aldrich); Ki-67 SP6, 1:200 (Neomarkers, Inc.); p27^{Kip1}, 1:250 (C-19, Santa Cruz Biotechnology, Inc.) and parafibromin, 1:50 (2H1, Santa Cruz Biotechnology)). Incubation with biotin-labelled secondary antibody (Biotinylated Goat Anti-Polivalent from Thermo Fisher Scientific, Inc.) was followed by revelation with 3,3-diaminobenzidine. The sections were counterstained with haematoxylin.

The anti-menin immunohistochemistry was performed with goat polyclonal antibody anti-menin (N-19 sc-8201; Santa Cruz Biotechnology). We used the Polink-2 Plus AP Goat Detection Kit (Golden Bridge International, Inc., Mukilteo, WA, USA) following the manufacturer's instructions. The immunohistochemical results were analysed according to the number of labelled cells, staining intensity, and protein subcellular location.

In all the experiments, positive and negative controls were used. The positive control was normal parathyroid tissue, except for cyclin D1 detection, where a breast cancer sample overexpressing this protein was used. The negative controls were performed by omission of the primary antibody.

Statistical analysis

In order to calculate the Hardy–Weinberg equilibrium, the Arlequin software (<http://anthro.unige.ch/arlequin/>) was used. Correlations were performed using Pearson correlation test, using SPSS 14.0 software. The level of statistical significance was set at $P < 0.05$.

Results

Study of germline mutations in MEN1, RET and CDKN1B genes

A search for germline mutations in *MEN1*, *RET* and *CDKN1B* genes was carried out. One patient had a germline heterozygous frameshift mutation (c.628_631delACAG) at exon 3 of the *MEN1* gene and was thereby excluded from this study. None of the 30 cases showed large *MEN1* germline deletions detected by MLPA and/or microsatellites polymorphic loci.

No other molecular alterations were detected at the germline level besides previously described, non-pathogenic DNA variants (Table 1). All the variants are in Hardy–Weinberg equilibrium and the genotypic and allelic frequencies found did not differ from those already described for European populations (<http://www.ncbi.nlm.nih.gov/snp>).

Table 1 Germline molecular alterations in *MEN1*, *RET* and *CDKN1B* genes ($n=30$).

Gene/exon	Genetic alteration classification	Nucleotide change	Genotypic frequencies (%)
<i>MEN1</i> /3	Deletion	c.628_631delACAG ^a	0.03
<i>MEN1</i> /9	Synonymous polymorphism	GAC → GAT (D418D)	CC=0.30 CT=0.57 TT=0.13
<i>RET</i> /11	Non-synonymous polymorphism	GGT → AGT (G691S)	GG=0.80 GA=0.17 AA=0.03
<i>RET</i> /13	Synonymous polymorphism	CTT → CTG (L769L)	TT=0.40 TG=0.57 GG=0.03
<i>RET</i> /14	Synonymous polymorphism	AGC → AGT (S836S)	CC=0.90 CT=0.10
<i>CDKN1B</i> /1	Non-synonymous polymorphism	GTC → GGC (V109G)	TT=0.80 TG=0.17 GG=0.03

^aMutation is numbered in relation to the *MEN1* cDNA reference sequence (GenBank accession number NM_130799.1), whereby nucleotide + 1 corresponds to the A of the ATG – translation initiation codon.

Somatic alterations in *MEN1* gene

Somatic mutations in the *MEN1* gene were found in two of the 24 parathyroid tumours (two adenomas: cases 3 and 17, Fig. 3). The mutations were located in exon 2; a four base pair deletion (c.249_252delGTCT) previously described (16), and a new mutation corresponding to a 49 bp deletion (c.115_163del49bp). Both mutations are frameshift deletions resulting in altered amino acid sequences that create premature stop codons at positions 117 and 102 respectively, presumably leading to a truncated protein. None of the mutations were found in the corresponding leukocyte DNA and were therefore considered somatic mutations.

In case 17, harbouring the somatic c.115_163del49bp deletion, the electropherogram analysis indicated that the tumour cells were hemizygous for the mutant allele suggesting LOH of the normal allele. This result was further confirmed by MLPA analysis that pointed to loss of most of the *MEN1* exons (Figs 1 and 3). As stated earlier, to exclude the presence of a germline deletion as the first hit (that could be missed by mutational analysis), the same methodology was applied to the corresponding leukocyte DNA. MLPA analysis of germline DNA did not show any sequence deletions, indicating that both events occur at the somatic level (Fig. 1).

Somatic intragenic deletions were further suspected in three additional cases (cases 1, 13 and 18), by analysis of the D418D polymorphic marker localised in exon 9 of *MEN1* gene. Those three patients, that present a heterozygous pattern (C/T) for the D418D variant in the germline, presented a hemizygous profile in the tumour DNA. These observations together with the identification of a somatic intragenic deletion by MPLA in case 17 led to the analysis of all the remaining tumours for LOH using MLPA. *MEN1* intragenic deletions were identified in seven additional cases, indicating the presence of LOH in 54% (13/24) of the

parathyroid tumours analysed; nine (69%) of these tumours were adenomas and the remaining four (31%) hyperplastic glands. Despite the different extents of these deletions, and their spread throughout the gene, they compromise mainly the terminal portion of the gene, being exons 2 and 3 maintained in most cases (Fig. 3). Results for cases 16 and 19 were done only once due to the scarcity of biological material. The tumours were further analysed for LOH using three microsatellites polymorphic loci from chromosome 11q13. All tissues were informative for at least one polymorphic marker.

In order to discard the possibility of MSI in the cases, that could lead to LOH misinterpretation, a set of five well-established quasimonomorphic microsatellites loci (29) were amplified in the tumours' DNA and none of them showed signs of MSI at any of the microsatellite loci analysed. We did not verify any correlation between the molecular alterations previously described and the histopathology of the lesions.

Genetic alterations in β -catenin and p27^{Kip1}

DNA sequencing analysis did not reveal any mutation in exon 3 of *CTNNB1* gene in the 24 studied tumours. The sequencing analysis of *CDKN1B* gene only revealed the presence of the variant already observed at the germline level, the V109G (SNP ID: rs2066827). The informative heterozygous genotypes in the germline were maintained at the somatic level, indicating retention of heterozygosity.

Immunohistochemical study of menin, β -catenin, p27^{Kip1}, cyclin D1, parafibromin and Ki-67

MEN1 mutations and/or deletions in parathyroid tumours are likely to result in a reduced (or absent)

Figure 1 Somatic inactivation of both *MEN1* alleles in case 17 leads to the absence of menin protein expression with consequent loss of immunoreactivity. As demonstrated in (A), using normal parathyroid tissue (wild type) as a reference, loss of menin expression was observed when compared with the tumour bearing *MEN1* mutation (mutated *MEN1*). The images are in the original magnification 200 $\times$ (upper images) and 400 $\times$ (lower images); (B) *MEN1* direct sequencing detected the presence of a deletion in the somatic counterpart (c.115_163del49bp mutated *MEN1*). The presence of the hemizygous deletion suggests inactivation of the other allele; (C) MLPA analysis confirmed the presence of a *MEN1* large deletion at the somatic level, leading to the ablation of the second allele. Such loss was not observed in the respective germline and the control case. Black arrows point to the exons detected to be lost by the MLPA probes. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-12-0327>.

expression of menin. Using an antibody against the N-terminal region of the protein, nuclear and cytoplasmic immunoreactivity was detected in all except one parathyroid tumour, with a variable number of immunoreactive cells. However, compared with normal parathyroid tissue, the tumours evidenced a clear down-regulation of this protein (Fig. 3).

β -Catenin staining evidenced a clear membrane pattern in all the samples and the intensity of the

staining ranged from weak to strong in 40–100% of the tumour cells (Tables 2, 3 and Fig. 2). Nuclear localisation of the protein was not observed in any case. All tumour samples expressed nuclear p27^{Kip1} protein staining, ranging from 14 to 98% of positive tumour cells (Table 2 and Fig. 3).

In nine tumours, no cyclin D1 expression was detected, where the remaining 15 tumours showed positive expression of varying intensity. In three of the positive cases (two adenomas and one hyperplastic gland), nuclear overexpression (in 20–35% of the tumour cells) was detected (Tables 2, 3 and Fig. 2).

In normal parathyroid tissue, parafibromin immunoreactivity displays a clear nuclear localisation. This staining pattern was maintained in all tumours that showed positivity in 70–100% of the tumour cells (Tables 2, 3 and Fig. 2). The 14 parathyroid tumours (12 adenomas and two hyperplastic glands) analysed for Ki-67 expression revealed low nuclear immunoreactivity (ranging from 0.02 to 4.2%) (Tables 2 and 3). No correlation was found between immunostaining with any of the above-mentioned antibodies and the molecular alterations detected in the respective tumours.

Discussion

The most common form of presentation of pHPT is as a sporadic disorder caused by parathyroid adenoma and less frequently by hyperplasia and carcinoma, but the molecular alterations underlying the sporadic forms remain largely unknown (30). In this study, we verify

Figure 2 Representative areas of parathyroid tumours, exemplifying immunostaining for β -catenin, cyclin D1, parafibromin and p27^{Kip1} proteins. All images are presented with an original magnification of 400 $\times$. From the immunohistochemistry results, we can observe: (A) β -catenin membranar immunostaining in 100% of the tumour cells; (B) overexpression of cyclin D1 protein in 20% of the parathyroid tumour cells; (C) intense parafibromin staining in 100% of the tumour cells; and (D) expression of p27^{Kip1} protein in about 62% of the tumour cells. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-12-0327>.

Table 2 Evaluation of protein expression levels in the series of the parathyroid tumours.

	Positive cases	Stained tumour cells (%)	Protein subcellular location
β-Catenin	24/24	40–100	Membranar
p27 ^{Kip1}	24/24	14–98	Nuclear and cytoplasmic
Cyclin D1	15/24	2–35	Nuclear
Parafibromin	24/24	70–100	Nuclear
Ki-67	14/14	0.02–4.2	Nuclear

that somatic intragenic deletions of *MEN1* gene appear as the most frequent genetic event in sporadic pHPT.

In order to exclude the family origin of the parathyroid tumours in our series, a search for germline mutations in *MEN1*, *RET* and *CDKN1B* genes was carried out and none of the cases corresponded to MEN2 or MEN4 syndromes. Germline studies for other familial syndromes, like familial hypocalciuric hypercalcaemia, neonatal severe HPT and HPT-JT, which can comprise pHPT as a clinical manifestation, were not performed due to the absence of familial and clinical data supporting such diagnoses.

The *MEN1* molecular studies report several types of *MEN1* mutations, which are scattered throughout the entire gene region. So far, neither hot spots have been described nor phenotype–genotype correlations have been established (31). About 40% of *MEN1* mutations are frameshift due to small deletions or insertions (18). *MEN1* large germline deletions compromise about 4% of the genetic defects (32), and as it has been previously reported (33, 34), they are only detected using gene dose assays. Despite their role in *MEN1* syndrome, mutations in this gene have also been identified in about 20% of familial isolated HPT cases (35, 36).

The D418D polymorphism detected in 70% of our series constitutes a relatively common polymorphism with no effects on protein (37). Correa *et al.* (38) demonstrated that *MEN1* D418D was associated with pHPT, representing a genetic risk factor for the disease. Although we have not evaluated the frequency of the polymorphism in a control series, the allelic frequencies found for D418D in pHPT patients are similar to those described for European control populations (<http://www.ncbi.nlm.nih.gov/snp>).

Two of the 24 tumours presented somatic mutations in *MEN1* gene: a 4 bp (c.249_252delGTCT) deletion without LOH and the other a 49 bp (c.115_163del49bp) deletion with loss of the wild-type allele. These frameshift mutations will lead either to truncated proteins with loss of functional domains, ablation of nuclear localisation signals in the C-terminal or result in loss of the translated protein due to nonsense-mediated mRNA decay (39). Additionally, it is known that both mutations occurred in a repetitive DNA sequence, consistent with a replication-slippage model of mutagenesis (18). In our series of parathyroid

tumours, 8% of the cases presented somatic *MEN1* mutations, which contrast with the 35% recently described by Newey *et al.* (40). At least two factors can explain these differences: on one hand, Newey *et al.* used whole-exome sequencing studies that can be much more sensitive, namely, detecting somatic mutations in heterogeneous tumours. On the other hand, the authors evaluated only uniglandular disease whereas 20% of our cases correspond to multiglandular disease (hyperplasia). The role of *MEN1* mutations in these two entities remains to be fully comprehended.

The finding of the hemizygous 115_163del49bp-mutated allele raised the hypothesis of a large germline deletion as the first hit that would have been missed by the conventional screening methods. The germline and somatic MLPA results enabled the exclusion of a germline large deletion, confirming the occurrence of the two inactivating events at the somatic level. These results were further confirmed with the microsatellite markers. The somatic biallelic inactivation detected seems to abolish menin expression as the immunostaining was lost in the tumour area (Fig. 1A), confirming the tumour suppressor role of *MEN1* in parathyroid tumorigenesis.

Taking into consideration the results obtained, a question was raised concerning the extent of such *MEN1* deletions in our series. Using MLPA assay both in germline and in somatic DNA, the presence of intragenic *MEN1* somatic deletions in 54% of the tumours was detected, pointing to *MEN1* intragenic deletions as the most frequent event in sporadic pHPT tumours. Therefore, two challenging observations have drawn our attention: the presence of discontinuous *MEN1* intragenic deletions and its possible role as a haploinsufficient gene in parathyroid tumorigenesis.

Gene deletions, either single-exon or partial/whole-gene deletions, are reported for several tumour suppressors namely in colorectal (*MLH1* and *MSH2*) (41), breast and ovarian cancers (*BRCA1* and *BRCA2*) (42). Additionally, it is known that Alu repeats are associated with genetic rearrangements that can create opportunities for unequal homologous recombination that can occur at intrachromosomal level, leading to gain or loss of exons in a gene (43). The plausibility of this hypothesis regarding *MEN1* gene was advanced by Fukuuchi *et al.* (44) that verify Alu repetitive elements scattered around *MEN1* gene, suggesting a role for Alu sequences in *MEN1* genetic deletions. Altogether, these data may support the discontinuity of *MEN1* intragenic deletions found in our work. Although MLPA analysis represents a powerful tool in genetic analysis being used in numerous studies (45, 46), the confirmation of the observed intragenic deletions would require the subcloning of PCR products in plasmids and further sequencing. These experiments could not be performed due to scarcity of tumour tissue and large size of deletion, making it difficult to obtain a reliable PCR

Age	Pathology	D11S956	PYGM	MLPA probes	RH27780	Mutations	MEN1 IHC (extension / intensity)
76	M		—				54 % / ++
87	M	—	—		—		100 % / +
77	A		—		—	c.249-252delGTCT	72% / ++
57	A				—		100% / +
70	A				—		64% / ++
61	A	—	—				28% / ++
77	A						100% / ++
54	A	—			—		62% / ++
53	A	—	—				25% / ++
32	A				—		32% / ++
62	A		—				100% / ++
47	A		—				40% / ++
60	M						35% / +
46	M		—				100% / ++
76	A		—				ND
68	A						21% / ++
58	A				—	c.115-163del49bp	0% / —
82	M				—		61% / ++
45	A		—		—		ND
50	A						41% / ++
72	A	—	—				29% / +
64	A	—					45% / ++
62	A	—			—		100% / ++
89	A		—		—		100% / ++

Legend:

Normal LOH with MLPA LOH with MLPA and LOH for D418D Ambiguous MLPA

— Non-informative marker

MLPA probes from exons 10 to 2

10	9	8	7	5	4	3	2
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+ Analysis of MLPA was only performed once.

A, adenoma; M, multiglandular hyperplasia;

ND – not determined.

Figure 3 Data on allelic deletions, mutations and expression of *MEN1* gene in the 24 parathyroid tumours.

product from FFPE. Also, the analysis by RT-PCR is not feasible, as RNA from the tumours is not available.

The other critical question is whether the observed *MEN1* intragenic deletions compromise one or both alleles. As mentioned earlier, MLPA is a powerful tool but is not able to discriminate if the observed discontinuous exonic deletions involve only one or both alleles. The fact that *MEN1* has been extensively described as a 'classical' tumour suppressor gene made us interpret our findings as intragenic biallelic inactivating deletions leading to protein down expression.

On the other hand, the hypothesis of monoallelic discontinuous intragenic deletions raises a critical question: the possibility that *MEN1* haploinsufficiency can be enough for parathyroid tumours development, which, as far as we are aware, was never described in pHPT. However, the discontinuity data regarding *MEN1* deletions presented in this study are preliminary and need further proof using independent methods.

The menin immunohistochemical results obtained in our work – all the cases, except one, evidence menin down expression compared with the only normal

Table 3 Immunohistochemistry results for β -catenin, p27^{Kip1}, cyclin D1, parafibromin, and Ki-67 proteins in the 24 parathyroid tumours.

Case	β -Catenin			p27 ^{Kip1}			Cyclin D1			Parafibromin			Ki-67	
	%	Loc	Loc	%	Int	Loc	%	Int	Loc	%	Int	Loc	%	Loc
1	40	++	M	68	++	N/C	2.7	+	N	75	+++	N	0.02	N
2	100	++	M	17	+	C	0.9	+	N	100	+++	N/C	ND	—
3	100	+++	M	94	+	N/C	0	—	—	100	++	N	1.0	N
4	100	+++	M	30	+	N/C	0.7	++	N	100	++	N	ND	—
5	100	+++	M	32	++	N/C	2.4	+++	N	100	+++	N	4.2	N
6	100	+++	M	50	+	N	0.7	+++	N	100	++	N	3.8	N
7	100	+++	M	30	++	N/C	19.7	+++	N	100	++	N	1.1	N
8	70	++	M	70	+	N/C	0.3	+++	N	100	++	N	ND	—
9	50	++	M	17.7	+	N	0.2	+	N	100	++	N	0	—
10	80	+++	M	15.4	++	N	0.8	++	N	70	+++	N	1.36	N
11	100	+++	M	40	+	N/C	2.5	+++	N	100	+++	N	0	—
12	100	+++	M	75.1	+++	N	3.8	++	N	100	++	N	ND	—
13	100	+++	M	98.2	+	N	0.2	++	N	100	+++	N	ND	—
14	100	+++	M	60	+	N	16.5	+++	N	100	+++	N	0	—
15	100	+++	M	72.9	+++	N/C	3.7	++	N	100	+++	N	ND	—
16	80	+++	M	14.2	+	N	1.1	++	N	80	++	N/C	1.7	N
17	40	++	M	30	++	N/C	1.79	+++	N	100	++	N	ND	—
18	100	+++	M	61.5	++	N/C	2.9	++	N	100	+++	N	ND	—
19	60	++	M	87.5	+++	N/C	35.3	+++	N	100	++	N	ND	—
20	70	++	M	18.5	++	N/C	1.6	++	N	70	++	N/C	3.8	N
21	100	+++	M	89.5	+++	N/C	2.2	+++	N	100	+++	N	1.4	N
22	100	+++	M	33	++	N	4.7	++	N	100	+++	N	2.2	N
23	100	++	M	88.5	+	N/C	0.4	+	N	100	+++	N	ND	—
24	100	+++	M	89.5	++	N/C	0.2	++	N	100	+++	N	1.5	N

%, Percentage of stained cells; Int, intensity; Loc, localisation; M, membranar staining; N, nuclear staining; C, cytoplasmic staining; ND, not determined; +, weak; ++, mild; +++, strong.

parathyroid tissue available – are compatible with *MEN1* inactivation. Our results are in agreement with Theodoropoulou *et al.*'s study where they verify *MEN1* LOH accompanied by weak protein expression (47).

Although the immunohistochemical results for menin are in line with MLPA data, protein down expression was also observed in cases without any *MEN1* genetic alterations. It is likely that additional mechanisms, independent of *MEN1* mutations or LOH, may contribute to menin down-regulation in parathyroid tumours. Disruption of promoter activity, either by hypermethylation or by mutations of regulatory elements, is a common inactivating mechanism of tumour suppressor genes. Previous works demonstrate (48) that *MEN1* promoter methylation can occur in pancreatic tumours, evidencing *MEN1* as a methylation-prone tumour suppressor gene. The consequence of CpG island hypermethylation is down-regulation of gene expression and it has been shown to function as a second hit event that leads to biallelic inactivation in some tumour suppressor genes (49, 50, 51, 52). Methylation of genes involved in parathyroid tumorigenesis has not been extensively studied, and the role of *MEN1* CpG hypermethylation remains to be clarified. Remarkably, Gao *et al.* (53) evidenced that *MEN1* promoter hypermethylation is responsible for menin down expression in melanoma cells. The hypothesis that disruption of *MEN1* promoter activity by mutation could constitute the 'second hit' in parathyroid tumours is plausible, but to our knowledge, the majority of the

works developed so far did not reveal any divergence from the wild-type promoter sequence. Only Jager *et al.* (54) described the presence of a *MEN1* regulatory mutation. These are important aspects to further elucidate the mechanism of *MEN1* inactivation.

To our knowledge, this is the first report describing *MEN1* intragenic deletions as a very frequent somatic event in parathyroid tumours in patients with sporadic pHPT. Further studies will be very relevant to verify the reproducibility of our findings and to analyse the biological effects of these intragenic deletions in pHPT.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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