A heterozygous frameshift mutation in exon 1 of CDKN1B gene in a patient affected by MEN4 syndrome

Francesco Tonelli, Francesco Giudici, Francesca Giusti1, Francesca Marini1, Luisella Cianferotti1, Gabriella Nesi1 and Maria Luisa Brandi1

Abstract

Objective: Multiple endocrine neoplasia type 4 (MEN4) is an autosomal dominant disorder that presents with a spectrum of clinical manifestations overlapping with those of MEN1 syndrome. It is caused by inactivating mutations of the CDKN1B gene, encoding for p27kip1 cyclin-dependent kinase 2 inhibitor, implicated in cell cycle control. Eight mutations of CDKN1B in MEN4 patients have been published so far. The aim of this study was to characterize the molecular basis of a case of MEN1-like syndrome with a neuroendocrine tumor and persistent primary hyperparathyroidism (PHPT).

Methods: Clinical, biochemical, and genetic evaluation were undertaken in the proband (a 53-year-old Caucasian woman) and in one 34-year-old son. The proband was operated for recurrent PHPT. Sequence analysis of the MEN1 and CDKN1B genes was performed on constitutional and parathyroid tissue DNA. Staining for p27 was carried out in parathyroid tissue.

Results: Neither MEN1 mutations nor large deletions encompassing the MEN1 gene on chromosome 11q13.1 could be detected in the proband. A germline frameshift mutation of CDKN1B (371delCT) was revealed, predicted to generate a truncated p27 (CDKN1B) protein. This mutation was confirmed on somatic DNA from the pathological parathyroid tissue, with the retention of the WT allele.

Conclusions: We report a germline heterozygote frameshift mutation of the CDKN1B gene in a Caucasian woman with a long clinical history of MEN1-like multiple endocrine tumors, along with the finding of the mutation in her son. This is the first report of positive CDKN1B mutation analysis in a male subject and also the first description of recurrent hyperparathyroidism in MEN4.

Introduction

Multiple endocrine neoplasia type 1 (MEN1, OMIM 131100, gene locus 613733 on chromosome 11q13.1) is a rare inherited cancer syndrome, characterized by the occurrence of tumors in at least two of the three main affected endocrine tissues (parathyroids, anterior pituitary, and duodenum–pancreas). Heterozygous germline mutations of the MEN1 oncosuppressor gene are identified in ~70% of patients with a recognized familial syndrome (1). Of the remaining 30% of patients with a MEN1-like clinical phenotype and in whom a MEN1 mutation was not found, about 1–3% are estimated to have germline large deletions within the gene that cannot be detected by the sequencing method, and few of the others can be suspected to bear mutations outside the MEN1-coding region and splicing sites, such as in regulatory sequences, promoter or intronic regions that are not currently investigated by genetic tests (2). For all the other patients, mutations in genes, other than MEN1, that can generate clinical phenocopies are to be suspected. The possibility of mutations of other genes involved in the pathogenesis of MEN1 syndrome has been widely investigated.
More than 10 years ago, a novel autosomal recessive multiple endocrine syndrome, named MENX, presenting a spectrum of tumors overlapping both MEN1 and MEN2 syndromes, has been associated with homozygote germline loss-of-function mutations of the Cdkn1b gene in rats (3). This gene encodes for the cyclin-dependent kinase (CDK) inhibitor p27kip1, that negatively regulates the progression of cellular mitosis during the G1-to-S phase transition by inhibiting the CDK2, thus controlling proliferation and also differentiation, cellular adhesion, and apoptosis (4). Expression studies demonstrated that expression of p27 (CDKN1B) protein is completely absent or extremely reduced in the normal and pathological tissues of these mutant rats. Studies in vitro on cell cultures have shown that the mutant p27 protein retained some property of the WT p27 protein, such as its localization to the nucleus and its interactions with CDK2, but is highly unstable and quickly degraded. The absence or the reduced levels of WT p27, a consequence of an altered synthesis of p27 or of its enhanced proteolysis, seemed to be responsible for the formation of endocrine tumors (4).

Mutation of the human homolog CDKN1B/p27kip1 gene in patients presenting with a MEN phenotype, but no germline mutations in the classical susceptibility genes MEN1 and RET, was found (4). Heterozygote inactivating mutations of the CDKN1B gene have been evidenced in <2% of patients screened for the presence of a MEN1 phenotype not harboring MEN1 gene mutations (1, 4, 5, 6, 7, 8, 9, 10), with a total of eight different germline mutations of the CDKN1B gene having been published so far (Table 1). This new syndrome, that in humans presents with an autosomal dominant pattern of inheritance, named MEN4 (4) (OMIM 610755, gene locus 600788 on chromosome 12p13.1), was associated with clinical, and genetic data and also for performing genetic tests both on blood- and parathyroid-derived DNA, and for expression and immunohistochemical studies on a parathyroid specimen. Informed consent for genetic tests was also obtained from the proband’s son (Fig. 1).

**Pedigree and family history**

The proband is a 53-year-old Italian woman of Caucasian ancestry. The father died from liver neoplasia at the age of 75 years. The mother died from rectal cancer at the age of 85 years. Neither of them presented with clinical manifestations suggestive for MEN1 syndrome. Two sisters and one brother did not display any sign of endocrine tumors or lesions associated with MEN1 syndrome. One sister, affected by diabetes mellitus, died from peritonitis at the age of 56 years. One 67-year-old brother suffered from hypertension and heart failure and one 69-year-old sister, who underwent hysterectomy, thyroidectomy, and cerebral aneurysm, refused to undergo biochemical and genetic tests for MEN1-associated endocrine alterations and tumors. The medical history of her three children was negative for any reported disease or apparent MEN1-associated clinical manifestation. Two out of three (age 35 and 28 years respectively) refused to undergo any investigation for biochemical or genetic MEN4 ascertainment, while the other (aged 34 years), otherwise asymptomatic, was tested for CDKN1B gene mutations.

**Mutation analysis of the MEN1 and CDKN1B genes**

Genomic DNA was extracted from peripheral blood leukocytes of the patient using a microvolume extraction method, NucleoSpin Blood Quick Pure (Macherey–Nagel, Easton, PA, USA), according to the manufacturer’s instructions. DNA was tested for quality by electrophoresis on 0.8% agarose gel and then quantified using a spectrophotometer.

Mutation analysis for the MEN1 gene was performed on genomic DNA as described previously (11). For the mutation analysis of the CDKN1B gene, coding regions (exons 1–2) and exon–intron junctions of the gene were amplified by PCR, using specific couples of primers located in the flanking intronic regions, in a 50 μl volume containing 50–100 ng DNA, 1 × PCR buffer, 2.5 mM MgCl₂, 0.25 mM deoxyribonucleotides, 0.4 μM of each primer, and one unit of Taq Polymerase. The PCR products were tested by 2% ethidium bromide-stained agarose gel electrophoresis and then purified by NucleoFast 96 PCR Plates for PCR product purification (Macherey–Nagel).

**Subjects and methods**

Informed consent was obtained from the patient for the collection, analysis, and publication of personal, familial, clinical, and genetic data.

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**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation Type</th>
<th>Location</th>
<th>Clinical Manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKN1B</td>
<td>5,8,9,10</td>
<td>Exons 1–2 and intron</td>
<td>MEN1-like</td>
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<tr>
<td></td>
<td></td>
<td>regions</td>
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**Fig. 1**

A new kindred with MEN4

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<table>
<thead>
<tr>
<th>References</th>
<th>CDKN1B/p27 mutation (its effect on p27 protein)</th>
<th>Sex</th>
<th>Clinical phenotype of proband (age at onset)</th>
<th>Family history (age at onset)</th>
<th>LOH for the CDKN1B/p27 gene mutation, tumor (yes/no)</th>
<th>Immunostaining for p27 protein, tumor (staining)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4)</td>
<td>Nonsense W76X (c.227G &gt; A) (p27 truncated at amino acid 76 with reduced expression of p27)</td>
<td>Female</td>
<td>GH-secreting pituitary adenoma (30 years) PHPT (46 years)</td>
<td>Father with acromegaly (not tested for p27 variant); two genetic carrier genders asymptomatic (44 years and teenager); and one positive female with renal angio myolipoma (55 years)</td>
<td>Renal angiomyolipoma (no)</td>
<td>Renal angiomyolipoma (no detection of staining)</td>
</tr>
<tr>
<td>(5)</td>
<td>Frameshift c.59_77dup19 (fsK25) (p27 protein 69 amino acids shorter than WT p27 and with a complete different amino acid sequence after codon 25)</td>
<td>Female</td>
<td>Small-cell neuroendocrine cervical carcinoma (45 years) ACTH-secreting pituitary adenoma (46 years) PHPT caused by one gland single adenoma (47 years)</td>
<td>First-degree relatives free from MEN1-related lesions</td>
<td>Cervical neuroendocrine carcinoma (yes)</td>
<td>Cervical neuroendocrine carcinoma (no detection of staining)</td>
</tr>
<tr>
<td>(1)</td>
<td>ATG-7G &gt; C in the 5’-UTR (reduced expression of p27)</td>
<td>Female</td>
<td>One HPT (one parathyroid gland) (61 years) Bilateral adrenal non-functioning mass (63 years) ZES and masses in duodenum and tail of pancreas (50 years)</td>
<td>Two asymptomatic daughters genetic carrier (47 and 48 years)</td>
<td>One parathyroid gland adenoma (no)</td>
<td>NA</td>
</tr>
<tr>
<td>(1)</td>
<td>Missense P95S (c.283C &gt; T) (p27 with a reduced capability to bind GRB2 regulatory protein)</td>
<td>Female</td>
<td>PHPT (two affected parathyroid glands) (50 years) ZES and masses in duodenum and tail of pancreas (50 years)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>(1)</td>
<td>STOP &gt; 199Q (c.595T &gt; C) (p27 with 60 amino acids longer than WT and reduced expression of p27)</td>
<td>Female</td>
<td>PHPT (three affected parathyroid glands) (50 years) Monozygotic twin sisters with PHPT (66 years) Two not genetically tested relatives with PHP: aunt and 52 years Female cousin (NA)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>(6)</td>
<td>P69L (c.678C &gt; T) (reduced expression of p27 and impairment in both binding CDK2 and inhibiting cell growth)</td>
<td>Female</td>
<td>Bilateral multiple lung metastatic bronchial carcinoid (67 years) PHPT (one affected parathyroid gland) (67 years) Papillary thyroid carcinoma with neck lymph node metastases (64 years) Subcutaneous epigastric lipoma (67 years) Non-functioning pituitary microadenoma (79 years)</td>
<td>Bronchial carcinoid (yes) Parathyroid adenoma (absent or reduced staining)</td>
<td>Bronchial carcinoid (no detection of staining)</td>
<td></td>
</tr>
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</table>
One aliquot of each purified PCR product was sequenced, both with forward and reverse primers, using the BigDye Terminator Purification Kit (Applied Biosystems) in a reaction consisting of 25 repeated cycles of denaturation for 10 s at 96°C, annealing for 5 s at 50°C, and extension for 2 min at 60°C. The sequencing products were then purified using the Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, Bedford, MA, USA) and analyzed on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequence images were obtained using the ABI Prism DNA Sequencing Analysis Software.

The obtained sequences were compared with a WT reference sequence for the human CDKN1B gene.

**Loss-of-heterozygosity analysis of the CDKN1B gene in a parathyroid lesion**

DNA was extracted from parathyroid hyperplastic tissue using the standard TRIzol protocol (Invitrogen, Life Technologies), according to the manufacturer’s instructions. DNA was then tested for quality by electrophoresis on 0.8% agarose gel and quantified using a spectrophotometer.

Coding regions (exons 1–2) and exon–intron junctions of the CDKN1B gene were sequenced, as described above for genomic DNA, in order to test the somatic heterozygote or hemizygote status of CDKN1B mutation in the pathological parathyroid tissue vs blood leucocytes. Sequence images were obtained using the ABI Prism DNA Sequencing Analysis Software (Fig. 2A and B).

**Expression analysis of CDKN1B mRNA in a parathyroid lesion**

Total mRNA was extracted from a parathyroid hyperplastic tissue sample using the standard TRIzol protocol according to the manufacturer’s instructions. An aliquot of total mRNA was retro-transcribed to cDNA and then amplified, using a specific couple of primers specifically designed between two different CDKN1B exons in order to amplify only RNA and not genomic DNA, to test if CDKN1B mRNA was expressed in parathyroid pathological tissue. A CDKN1B non-mutated parathyroid adenoma was used as a control. PCR products were verified by electrophoresis on agarose gel 2% and expression images were acquired using the BioDoc-it Imaging System (UPV, Upland, CA, USA; Fig. 3A). The CDKN1B expression values were calculated using the ImageJ Software (free software available at http://imagej.nih.gov/ij/), using the expression of 18S, which is treated as a housekeeping gene (Fig. 3B).
The expression values of CDKN1B were also measured by quantitative real-time RT-PCR in the CDKN1B-mutated hyperplastic parathyroid (case report) with respect to one normal parathyroid, one sporadic hyperplastic parathyroid negative for both CDKN1B and MEN1 mutations, two sporadic parathyroid adenomas negative for both CDKN1B and MEN1 mutations, one MEN1 parathyroid with a recognized MEN1 mutation, and one sporadic parathyroid carcinoma negative for both CDKN1B and MEN1 mutations. From each sample, 20 ng of total RNA were obtained and used for the expression analysis, using the same couple of primers of the CDKN1B semi-quantitative PCR expression analysis described above, the Brilliant II SYBR Green QRT-PCR Kit, and the MX300p instrument (Stratagene, Agilent Technologies, Santa Clara, CA, USA), according to the manufacturers’ instructions. The level of expression of CDKN1B in the case report was used as a comparative calibrator, while β-actin was used as an expression-normalizing housekeeping gene. All the samples were analyzed in triplicate and differences in expression, with respect to the case report, were valued by the Student’s t-test.

Moreover, the region of cDNA containing the CDKN1B mutation was PCR amplified, using a specific couple of primers not encompassing the region of the mutation, and then sequenced, as described above for genomic and somatic DNA, to verify if a WT or a mutated allele or both were expressed in the parathyroid lesion of the patient. Sequence images were obtained using the ABI Prism DNA Sequencing Analysis Software (Fig. 2C and D).

### Immunohistochemistry

The specimen was fixed in 10% formalin before being processed in paraffin. Representative sections of the lesions were selected for immunohistochemical analysis. We used mouse monoclonal anti-p27kip1 (clone SX53G8, Ventana, Tucson, AZ, USA) and rabbit monoclonal CONFIRM anti-Ki-67 (clone 30-9, Ventana) as primary antibodies. The tissue sections were placed on the automated staining system BenchMark XT (Ventana Medical Systems) within which samples were deparaffinized, rehydrated, and processed for blocking endogenous peroxidase and epitope retrieval. Primary antibodies were incubated according to the protocol suggested by Ventana. The UltraView Universal DAB Detection Kit (Ventana) was used as the revelation system. Upon

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**Figure 1**
Schematic of the pedigree (proband with MEN1 phenotype: black filled symbol pointed out by the arrow; proband’s son, positive for CDKN1B mutation but negative for MEN1 clinical manifestations: gray symbol).

**Figure 2**
Sequencing of the CDKN1B gene. (A and B) The forward and reverse sequences of CDKN1B mutation in genomic DNA from the venous blood sample of the patient. (C and D) The forward and reverse sequences of CDKN1B mutation in somatic DNA from the hyperplastic parathyroid tissue of the CDKN1B-mutated patient. Sequences evidenced the presence of both mutated and WT alleles in the pathological tissue. (E and F) The forward and reverse sequences of CDKN1B mutation in mRNA (reverse transcribed to cDNA) from the hyperplastic parathyroid tissue of the CDKN1B-mutated patient. Sequences evidenced the presence (expression) of only WT CDKN1B mRNA, and not the mutated one, in the pathological tissue. Black arrows indicate the position of the nucleotide’s mutation.
completion of the staining process, tissue sections were removed from the automated staining system and counterstained with Mayer’s hematoxylin.

Positive controls encompassed tonsil for Ki-67 and colon for p27. The primary antibody was substituted with a non-immune serum at the same concentration and this was used as a negative control. The control sections were treated in parallel with the samples. All sections were dehydrated and mounted with the Permount mounting medium.

Results

Description of the case

At the age of 33 years, the patient underwent a tubaric pregnancy that required salpingectomy. At the age of 41 years, she developed symptoms of hypothyroidism due to Hashimoto’s thyroiditis. In the same year, primary hyperparathyroidism (PHPT) was diagnosed, with consequent surgical ablation of two enlarged parathyroid glands (left and right inferior; 10×8×4 and 40×25×20 mm respectively). Both glands were histologically parathyroid adenomas. After surgery, calcium levels became normalized, with persistence of PHPT. At the age of 43 years, she underwent hysterecrotomy for a fibroma. At the age of 48 years, owing to chronic abdominal pain and recurrent obstructive symptoms, adhesionolysis was performed, ileocecal appendix was removed, and incisional hernia was repaired. At the age of 50 years, the patient suffered epigastric symptoms. Esophagogastroduodenoscopy showed two small ulcers in the second duodenal portion. Increased plasma levels of chromogranin A (CgA) and basal gastrin were detected. Both abdominal computerized tomography (CT) scan and magnetic resonance imaging (MRI) visualized a node (1.5 cm in diameter) near the pancreatic head. Somatostatin receptor scintigraphy (OctreoScan; SSRS) resulted positive for the presence of two increased uptake areas, one in the pancreatic head and the other across the V–VI hepatic segments. These areas were both diagnosed as neuroendocrine tumors of the pancreas with suspected liver metastases. Tumors were treated with proton pump inhibitors and octreotide until resolution of symptoms (September 2011). A following abdominal MRI did not evidence any pancreatic lesion, but an enlarged retro-pancreatic lymph node (1.5 cm in diameter). After 1 year, the abdominal CT showed focal enhancement of the middle duodenal wall and confirmed the presence of the increased lymph node behind the pancreatic head. Somatostatin analog therapy was restored. At the age of 50 years, the patient underwent cervical reoperation to treat the persistent PHPT with the removal of an increased right superior parathyroid gland. At the age of 51 years, the patient was admitted to the emergency room because of acute abdominal pain caused by an ulcer perforation at the third duodenal portion. Gastro-duodenal resection with duodenal–jejunal and gastro-jejunal anastomoses were performed, with the removal of the gallbladder. Two large periduodenal nodes were detected and histologically identified as low-grade, well-differentiated neuroendocrine tumors (Ki-67 growth marker <2%). No liver metastases were identified during surgery. Treatment with octreotide was maintained, with periodical and specific biochemical and imaging follow-ups. Serum CgA was not normalized (43 nmol/l and n.v. 0–6 nmol/l), although SSRS was completely negative. CT and MRI were negative for focal lesions. Gallium 68-labeled positron emission tomography showed pathological uptake at the level of the right petrous apex. At the age of 52 years, the patient complained of colic symptoms at the abdominal level with
a further increase in serum CgA levels (465 nmol/l). A new abdominal CT scan showed a node (7 mm in diameter) within the middle duodenal portion associated with enhancement in the arterial phase. Owing to the persistence of the chronic abdominal pain, an additional duodenopancreatectomy was performed. The histopathological examination showed multiple small (<0.5 cm) well-differentiated neuroendocrine neoplasias in the duodenal wall, with 1% Ki-67, <2×50 high power field (HPF) mitoses, and no signs of angioinvasivity or necrosis. Immunohistochemistry was positive for CgA, synaptophysin, and CD56 and negative for gastrin or other pancreatic neuropeptides. The pancreatic parenchyma was free from lesions. Metastatic tissue was present in one out of 33 lymph nodes examined.

In April 2012 (55 years of age), the patient was referred to the Tuscany Regional Center for Hereditary Endocrine Tumors in Florence. According to her clinical history, characterized by a likely persistence/recurrence of PHPT (high PTH levels in the presence of 25-OH vitamin D above 30 ng/ml) and neuroendocrine tumors of the gastro-entero-pancreatic tract, the presence of MEN1 syndrome was suspected. Biochemical serum examinations confirmed the increased values of parathyroid hormones, with ionized calcium level in the upper normal range. The secretin stimulation test did not show any pathological increase in the serum levels of entero-pancreatic endocrine peptides. Prolactin levels were found to be within the normal range (462 and 391 mU/l at 0 and +30’, n.v. 72–504). MRI showed asymmetry of the pituitary gland (left > right), without focal abnormalities. Pituitary functionality tests were normal (follicle-stimulating hormone, 72.57 U/l (normal range 26–138); luteinizing hormone, 38.34 U/l (n.v. 16–64); adrenocorticotropic hormone (ACTH), 17.1 ng/l (n.v. 9–52); growth hormone (GH), 0.32 µmol/l (n.v. 0.8–5.4); thyroid-stimulating hormone, 2.22 mU/l (n.v. 0.25–3.5); and insulin-like growth factor 1, 169 ng/ml (n.v. 81–225)). Lumbar spine (L1–L4) dual energy X-ray absorptiometry (DXA) showed osteoporosis with a T-score of −2.8 (0.806 g/cm²), with osteopenia at the femoral neck with a T-score of −1.9 (0.706 g/cm²). The patient underwent mutation analysis for both the MEN1 and CDKN1B genes.

In November 2012, the patient was referred to the Florence Surgical Unit for the treatment of persistent PHPT complicated with osteoporosis. Before surgery, the patient underwent Sestamibi scanning that revealed a pathological uptake besides the left thyroid lobe, although the ultrasound was negative. During surgery (November 2012), the left superior parathyroid gland was found in its
typical site but with increased volume (19×9×4 mm) with a polylobulated shape. The intraoperative monitoring of PTH showed a decrease in PTH level to undetectable values (<1.3 pmol/l) 60 min after parathyroidectomy of the left superior gland, confirming the removal of all functioning parathyroid tissues from the cervical region and excluding the presence of supernumerary and/or ectopic gland. Eight fresh non-adenomatous parathyroid gland fragments (each of about 1 mm in diameter) were autografted in the subcutaneous tissue of the brachioradialis muscle of the non-dominant forearm at the same time as the parathyroidectomy intervention. A recurrent meso-epigastric incisional hernia was also repaired during the same operation by laparotomy, which allowed the exploration of the abdominal cavity and confirmed the negativity of preoperative examinations for recurrent endocrine tumors of the entero-gastropancreatic tract and the liver. Histopathological evaluation of excised parathyroid identified clear cell foci of parathyroid hyperplasia within the principal cells of the gland itself. A sample of the pathological parathyroid was collected in RNAlater and sent to the laboratory for loss-of-heterozygosity (LOH) evaluation.

Calcium gluconate i.v. infusion (two ampoules, 10%) was required during the first and second postoperative days to treat the hypoparathyroidism (serum calcium, 7.6 mg/dl). The postoperative course was uneventful and the patient was discharged during the fifth postoperative day with oral calcium and calcitriol supplements. Long-term postoperative blood tests showed permanent hypoparathyroidism, which has been treated with oral calcium, calcitriol, and cholecalciferol. After discontinuation of calcitriol, the patient showed asymptomatic hypocalcemia. Approximately 1 year after surgery, therapy with calcitriol was discontinued, while still maintaining calcium and cholecalciferol supplementation. Under this new regimen, the patient was asymptomatic, still showing mild hypocalcemia (7.7 mg/dl). In the follow-up, the patient also underwent periodical biochemical and hormonal testing according to the clinical guidelines. As prolactin levels were high (1165 mU/ml) in the absence of any medication that could affect its levels (i.e. H2 blockers or somatostatin analogs) or kidney/liver functional abnormalities, she was placed under dopamine agonists, with normalization of prolactin values (75 mU/ml) at 1-year follow-up.

**Genetic analyses**

Genetic analyses in the proband showed lack of MEN1 gene mutations either in the coding region or in the
intron–exon junctions. Large deletion encompassing MEN1 locus on chromosome 11q13.1 was excluded. Mutational analysis of the CDKN1B gene on leukocyte genomic DNA revealed the presence of a novel heterozygous frameshift 371delCT mutation at codon 125 in exon 1. The presence of this deletion generates a slippage of the genetic code that creates a premature ATG stop codon at codon 145, with a subsequent probable synthesis of a truncated p27 protein.

The same mutation was also found, in heterozygous status, in the blood-derived genomic DNA from the patient’s 35-year-old son. He has not yet presented with any MEN4-associated clinical features, with the exception of calcemia in the upper normal range (serum calcium, 9.5 mg/dl; ionized calcium, 5.26 mg/dl; fosfatemia, 2.7 mg/dl; PTH, 6.4 pmol/l; and PRL, 162–122 mU/ml).

Genetic analysis of the CDKN1B sequence on somatic DNA from the hyperplastic parathyroid tissue confirmed the presence of the 371delCT mutation (Fig. 2A and B). The mutation was found in heterozygous DNA from the patient’s 35-year-old son. He has not yet presented with any MEN4-associated clinical features, with the exception of calcemia in the upper normal range (serum calcium, 9.5 mg/dl; ionized calcium, 5.26 mg/dl; fosfatemia, 2.7 mg/dl; PTH, 6.4 pmol/l; and PRL, 162–122 mU/ml).

CDKN1B mRNA was expressed in the CDKN1B-mutated parathyroid hyperplastic tissue in greater levels than in the adenomatous CDKN1B non-mutated parathyroid tissue (obtained from a patient with sporadic PHPT and used as a control) with only the WT allele of CDKN1B being expressed, as revealed by DNA sequencing of cDNA (Fig. 3).

CDKN1B mRNA was expressed in the CDKN1B-mutated parathyroid hyperplastic tissue (Fig. 3). Interestingly, the expression of CDKN1B was significantly (P<0.01) higher than in a normal parathyroid sample, one sporadic hyperplastic parathyroid, two sporadic parathyroid adenomas, one MEN1 parathyroid adenoma, and one sporadic parathyroid carcinoma (Fig. 4). Moreover, only the WT allele of CDKN1B was expressed in our patients as revealed by DNA sequencing of cDNA (Fig. 2E and F).

On histological examination, the parathyroid tissue consisted predominantly of chief cells distributed in a nodular pattern, but foci of clear cells with well-defined cytoplasmic membranes were also present (Fig. 5). Stromal fat cells were markedly decreased.

In immunohistochemistry analyses, parathyroid cells exhibited reduced nuclear p27 staining when compared with the interspersed normal endothelial cells. There was virtually a lack of Ki-67 expression in parathyroid cells (Fig. 5).

Discussion

MEN syndromes are autosomal dominant rare disorders characterized by the development of tumors in two or more endocrine tissues. The two main MEN syndromes are MEN1 caused by germline loss-of-function mutations of the MEN1 oncosuppressor gene (12) and MEN2, caused by germline gain-of-function mutations of the RET proto-oncogene (13).

More than 6 years ago, the autosomal dominant MEN4 syndrome was described for the first time in humans and referred to mutations of the CDKN1B gene (4). Following this original description, other patients presenting with a MEN1 phenotype, but no germline mutations in the MEN1 gene, were described (1, 4, 10). In the evaluated cases, CDKN1B gene mutations generate a decrease in its encoded product, through various mechanisms, encompassing the synthesis of a truncated protein and/or the reduction of either protein expression
or protein life, as confirmed by immunohistochemical studies showing complete absence or strong reduction in p27 expression in tumoral tissues or its delocalization from the nucleus to the cytoplasm (4, 5, 6, 8). One of the identified mutations consists of a nucleotide variation at a stop codon associated with the formation of a protein longer than normal, mostly localized in the cytoplasm rather than in the nucleus, with a low stability and a reduced CDK2 binding affinity (1).

In normal conditions, p27 expression is regulated by different mechanisms: transcriptional, translational, and post-translational. Expression and functional studies seem to suggest that, in MEN4 tumors, p27 acts as a non-canonical haplo-insufficient tumor suppressor gene. A mutated transcript can determine a truncated protein or an altered protein mainly localized in the cytoplasm rather than in the nucleus with a lower stability and a diminished CDK2 binding property (6). During MEN4 tumorigenesis, the principal mechanism responsible for the reduced expression of p27 seems to be the post-translational ubiquitin–proteasome degradation of the protein (1, 7, 14) and only in rare cases the reduced p27 expression is ascribable to CDKN1B somatic mutations or tissutal somatic CDKN1B LOH (5, 6). The p27 protein mainly localized to the cytoplasm is rapidly degraded through ubiquitylation by the KPC ubiquitin ligase and proteasome-mediated degradation (15, 16, 17) in tumors, which is different from the normal conditions. In MEN4 syndrome, unlike MEN1, somatic LOH seems to be a rare event, even though only few MEN4 endocrine tumors have been examined for somatic LOH, which has never been found in the parathyroid gland adenomas. Somatic LOH has only been found in a cervical neuroendocrine metastatic carcinoma and in a bronchial metastatic carcinoid (5, 6), suggesting that LOH in MEN4 syndrome could be associated with the aggressive and malignant progression of the neoplasia.

Herein, we describe a case of MEN4 syndrome with various and recurrent endocrine tumors of the parathyroid glands and gastro-entero-pancreatic tract, associated with a novel CDKN1B mutation, consisting of a heterozygous deletion of CT residues at position 371 in exon 1 that generates a premature stop at codon 125 and possibly a truncated p27 protein. No somatic LOH has been found in the parathyroid hyperplastic tissue, confirming data of previously published studies (1, 6), with only a WT CDKN1B mRNA allele being expressed at the mRNA level, a significantly higher expression of CDKN1B mRNA and reduced nuclear p27 staining. Altogether, these results suggest that, in this case, the down-regulation of p27 protein expression could be at a post-transcriptional and/or post-translational level.

Interestingly, all MEN4 patients, described to date, showed a relatively late age of clinical expression when compared with MEN1 patients. PHPT has a high penetrance in MEN4 and, similar to MEN1, is the first diagnosed endocrinopathy in most cases, but with an age at onset more than two decades later than in MEN1 (mean 56 years in MEN4 vs 20–25 years in MEN1) (1, 4, 5, 6, 7, 8). In about half of the MEN4 patients, only one gland was affected and responsible for PHPT, without geno-type/phenotype relationships ever described regarding the severity and the number of the involved parathyroids (1, 4, 5, 6, 7, 8). The follow-up of these patients is often lacking, and no information on the persistence or recurrence of PHPT is usually referred. The strict follow-up of the present case made it possible to document for the first time a progressive tumoral involvement of all four parathyroids, causing persistent PHPT with full resolution of the disease only after removal of all the parathyroid tissues.

For its frequency, anterior pituitary adenoma is the second manifestation of MEN4 syndrome. Both functioning (GH or ACTH) and non-functioning tumors have been observed (4, 5, 6, 7, 8). The present case showed an increase in the values of prolactin without relevant morphological evidence of a lesion at the pituitary level.
Only one of the eight described MEN4 patients showed duodenum–pancreatic endocrine tumors, responsible for Zollinger–Ellison Syndrome (ZES), with progressive multiple endocrine involvement of the pancreas and the duodenum ascribed to gastrinomas (1).

A positive family history was described only in three MEN4 cases and in these cases the relatives, inheriting the CDKN1B mutation, exhibited an attenuated form of the disease (1, 4). In the present case, the only tested first-degree relative, at 35 years of age, was found to bear the same mutation of his mother, without any clinical manifestation or sign of MEN4-related endocrine dysfunctions. These findings support the late onset of the disease and will make it possible to follow the natural history of this disorder in an asymptomatic carrier.

Interestingly, all patients affected by MEN4 are women and the great majority of clinical manifestations have arisen around the age of menopause (1, 5) or later (1, 6, 7), even in their mutated female first-degree relatives (1). No mutated male patients or relatives have been reported to date, except for the son of this index case, who does not present with MEN4-associated clinical manifestations, so far. This particular feature suggests a possible effect of sexual hormones on p27 stability and activity or genomic imprinting. The effects of estrogens and progesterone on p27 have been investigated in normal and cancer endometrial cells, showing that these hormones regulate the nuclear level of p27 in an opposite manner (18). Estrogens down-regulate the levels of p27 through the increase in its ubiquitin–proteasome-mediated degradation, meanwhile progesterone up-regulates p27 through the inhibition of the same degradation system (18). Moreover, possible interactions between androgens and p27 have been investigated both in ovarian and in breast tumor cells, demonstrating that dihydrotestosterone down-regulates p27 through the ubiquitin–proteasome system by inducing the direct binding of p27 to SKP2 ubiquitin ligase, independent of the p27 phosphorylation status (19). The enhanced proteolysis of p27, mediated by sexual hormones, could be a susceptibility co-factor also for tumorigenesis in MEN4 syndrome. However, the role of these hormones in MEN4 remains to be investigated in normal and tumoral tissues, classically affected by MEN4, as well as in normal and tumoral cells from CDKN1B-mutated subjects.

The phenotypic expression of MEN4 is still not completely defined due to the limited number of MEN4 patients described until now, each having a different CDKN1B gene mutation (1, 4, 5, 6, 7, 8). This makes it difficult to uncover the genotype/phenotype correlations, useful to draw a preventing screening protocol. Therefore, it is of great importance for the endocrine community to document in detail any new MEN4 case/pedigree.

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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