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

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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 p27^{kip1} 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.

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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 p27^{kip1}, 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/p27^{kip1}* 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), to distinguish it from MEN1, MEN2A (OMIM 171400, gene locus 164761 on chromosome 10q11.21), and MEN2B (formerly MEN3) (OMIM 162300, gene locus 164761 on chromosome 10q11.21), exhibits MEN1-like clinical manifestations but not MEN2-associated tumors in all the reported conditions (Table 1) (1, 4, 5, 6, 7, 8, 9, 10).

In this paper, we report a case of a Caucasian female patient with a long clinical history suggestive of a MEN1 syndrome, but with a negative *MEN1* genetic test, in which a novel germline heterozygote frameshift mutation of the *CDKN1B* gene has been identified.

Subjects and methods

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

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).

Table 1 Phenotype and genotype of published MEN4 patients.

References	CDKN1B/p27 mutation (its effect on p27 protein)	Sex	Clinical phenotype of proband (age at onset)	Family history (age at onset)	LOH for the CDKN1B/p27 gene mutation, tumor (yes/no)	Immunostaining for p27 protein, tumor (staining)
(4)	Nonsense W76X (c.227G>A) (p27 truncated at amino acids 76 with reduced expression of p27)	Female	GH-secreting pituitary adenoma (30 years) PHPT (46 years)	Father with acromegaly (not tested for p27 variant); two genetic carrier genders asymptomatic (44 years and teenager); and one positive female with renal angiomyolipoma (55 years)	Renal angiomyolipoma (no)	Renal angiomyolipoma (no detection of staining)
(5)	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)	Female	Small-cell neuroendocrine cervical carcinoma (45 years) ACTH-secreting pituitary adenoma (46 years) PHPT caused by one gland single adenoma (47 years) One HPT (one parathyroid gland) (61 years)	First-degree relatives free from MEN1-related lesions	Cervical neuroendocrine carcinoma (yes)	Cervical neuroendocrine carcinoma (no detection of staining)
(1)	ATG-7G>C in the 5'-UTR (reduced expression of p27)	Female	Bilateral adrenal non-functioning mass (63 years) PHPT (two affected parathyroid glands) (50 years) ZES and masses in duodenum and tail of pancreas (50 years)	Two asymptomatic daughters genetic carrier (47 and 48 years)	One parathyroid gland adenoma (no)	NA
(1)	Missense P95S (c.283C>T) (p27 with a reduced capability to bind GRB2 regulatory protein)	Female	PHPT (three affected parathyroid glands) (50 years)	NA	NA	NA
(1)	STOP>199Q (c.595T>C) (p27 with 60 amino acids longer than WT and reduced expression of p27)	Female	PHPT (three affected parathyroid glands) (50 years)	Monozygotic twin sisters with PHPT (66 years) Two not genetically tested relatives with PHP: aunt (52 years) Female cousin (NA)	NA	NA
(6)	P69L (c.678C>T) (reduced expression of p27 and impairment in both binding CDK2 and inhibiting cell growth)	Female	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)	NA	Bronchial carcinoid (yes) Parathyroid adenoma (no)	Parathyroid adenoma (absent or reduced staining) Bronchial carcinoid (no detection of staining)

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Table 1 Continued

References	CDKN1B/p27 mutation (its effect on p27 protein)	Sex	Clinical phenotype of proband (age at onset)	Family history (age at onset)	LOH for the CDKN1B/p27 gene mutation, tumor (yes/no)	Immunostaining for p27 protein, tumor (staining)
(7)	GAGA deletion in 5'-UTR (c. -32_ -29delGAGA) (reduced expression of p27)	Female	Gastric carcinoid (69 years) PHPT (74 years)	NA	NA	NA
(8)	CCTT deletion in 5'-UTR (c. -456_ -453del CCTT) (deletion shifts the ORF, altering the normal protein translation from the main ATG and resulting in a significant reduction in p27 expression)	Female	GH pituitary adenoma (NA) Well-differentiated non- functioning pancreatic neoplasia (NA)	NA	Endocrine pancreatic tumor (no)	Endocrine pancreatic tumor (weak nuclear and cytoplasmic staining)

PHPT, primary hyperparathyroidism; NA, not available; ORF, open reading frame.

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).

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).

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-p27^{kip1} (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

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.

Figure 3

Expression of *CDKN1B* mRNA in the *CDKN1B*-mutated parathyroid lesion and in a *CDKN1B* non-mutated parathyroid adenoma. (A) The result of *CDKN1B* mRNA PCR amplifications in the *CDKN1B*-mutated parathyroid lesion and *CDKN1B* non-mutated parathyroid adenoma as a control. (B) The graphical representation of the *CDKN1B*/*S18* expression ratio in the *CDKN1B*-mutated parathyroid lesion and *CDKN1B* non-mutated parathyroid adenoma.

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 hysterectomy for a fibroma. At the age of 48 years, owing to chronic abdominal pain and recurrent obstructive symptoms, adhesiolysis 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); adrenocorticotrophic 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 status, indicating the absence of *CDKN1B* LOH at somatic level, with the retention of the WT copy of *CDKN1B* in the hyperplastic parathyroid tissue (Fig. 2C and D).

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 a 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).

Figure 4

Graphical representation of the quantitative expression of *CDKN1B* mRNA, evaluated by quantitative real-time RT-PCR on total RNA extract, in a panel of healthy and pathological parathyroid samples with respect to the *CDKN1B*-mutated hyperplastic parathyroid case report (1, normal parathyroid tissue; 2, sporadic hyperplastic parathyroid negative for *MEN1* and *CDKN1B* mutations; 3, no. 1 sporadic parathyroid adenoma negative for *MEN1* and *CDKN1B* mutations; 4, no. 2 sporadic parathyroid adenoma negative for *MEN1* and *CDKN1B* mutations; 5, *MEN1* parathyroid adenoma; 6, *CDKN1B*-positive proband; and 7, sporadic parathyroid carcinoma negative for *MEN1* and *CDKN1B* mutations). *Indicates a significant different expression relative to sample 6, evaluated by the Student's *t*-test ($P<0.01$).

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

Figure 5

Histological phenotype of the pathological parathyroid tissue. (A) Hematoxylin–eosin coloration of the pathological parathyroid tissue from the proband (original magnification, 50×). The pathological tissue showed a nodular growth pattern with foci of clear cells. Fat cells were identified as single cells scattered throughout the parenchyma. (B) Immunohistochemistry for p27 protein in pathological parathyroid tissue of the proband (original magnification, 100×). Nuclear immunoreactivity for p27 was markedly reduced with respect to normal endothelial cells, positive for p27 and serving as an internal control (inset). (C) Ki-67 coloration of the pathological parathyroid tissue from the proband (original magnification, 100×). Nuclear expression of Ki-67-positive cells was negligible.

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 genotype/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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