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 CDKN1B gene, encoding for p27kip1 cyclin-dependent kinase 2 inhibitor, implicated in cell cycle control. Eight mutations of CDKN1B have been published in MEN4 patients. 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.

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

Results No MEN1 mutations nor large deletions encompassing 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 protein. This mutation was confirmed on somatic DNA from the pathological parathyroid tissue, with the retention of the wild type allele.

Conclusions We report a germline heterozygote frameshift mutation of 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 approximately 70% of the 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 to homozygote germline loss-of-function mutations of the *Cdkn1b* gene in rats [3]. This gene encodes for the cyclin-dependent kinase inhibitor p27kip1, that negatively regulates the progression of cellular mitosis during the G1-to-S phase transition by inhibiting the cyclin-dependent kinase 2 (CDK2), thus, controlling proliferation and also differentiation, cellular adhesion and apoptosis [4]. Expression studies demonstrated that expression of p27 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 wild-type 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 wild-type p27,
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 homologue CDKN1B/p27kip1 gene in patients presenting a MEN phenotype, but no germline mutations in the classical susceptibility genes MEN1 and RET, was found [4]. Heterozygote inactivating mutations of CDKN1B gene have been evidenced in less than 2% of the patients screened for the presence of a MEN1 phenotype not harboring MEN1 gene mutations [1,4-10], with a total of eight different germline mutations of CDKN1B gene having been published (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) [1,4-10].

In this paper, we report a case of an 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 parathyroid specimen. Informed consent for genetic test was obtained also by the proband’s son (Fig. 1).

Pedigree and family history

The proband is a 53 years old Italian woman of Caucasian ancestry. The father died for liver neoplasia at age 75. The mother died for rectal cancer at age 85. Neither of them presented clinical
manifestations suggestive for MEN1 syndrome. Two sisters and one brother did not display any
sign of endocrine tumors or lesions associated to MEN1 syndrome. One sister, affected by diabetes
mellitus, died at age 56 for peritonitis. 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 (age 34 years), otherwise asymptomatic, was tested for CDKN1B gene mutations.

Mutation Analysis of 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 by spectrophotometer.

Mutation analysis for the MEN1 gene was performed on genomic DNA as previously
described [11].

For the mutation analysis of CDKN1B gene, coding regions (exons 1-2) and exon-intron
junctions of the gene were amplified by PCR reaction, using specific couples of primers located in
the flanking intronic regions, in a 50 µl volume containing 50-100 ng of DNA, 1X PCR buffer,
2.5mM 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, Easton, PA, USA). One aliquot of each purified PCR product was sequenced,
both with forward and reverse primers, using the BigDye Terminator Purification Kit (Applied

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Biosystems, Foster City, CA, USA) in a reaction consisting of twenty-five repeated cycles of denaturation for 10 sec at 96°C, annealing for 5 sec at 50°C and extension for 2 min at 60°C. The sequencing products were then purified with Montage SEQ96, Sequencing Reaction Cleanup Kit (Millipore, Bedford, MA, USA) and analyzed on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence imaging were obtained by ABI Prism DNA Sequencing Analysis Software.

The obtained sequences were compared to wild type reference sequence for the human CDKN1B gene.

Loss of heterozygosity analysis of CDKN1B gene in parathyroid lesion

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

Coding regions (exons 1-2) and exon-intron junctions of 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 imaging were obtained by ABI Prism DNA Sequencing Analysis Software (Fig. 2A, 2B).

Expression analysis of CDKN1B mRNA in parathyroid lesion

Total mRNA was extracted from parathyroid hyperplastic tissue sample using 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 control. PCR products were verified by electrophoresis on agarose gel 2% and expression images were acquired by BioDoc-it Imaging System (UPV, Upland, California, USA) (Fig. 3A). The CDKN1B expression values were calculated with Image J software, using the expression of 18S as 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) 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. Twenty nanograms of total RNA from each sample were used for the expression analysis, using the same couple of primes 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 comparative calibrator, while beta-actin was used as expression normalizing housekeeping gene. All the samples were analyzed in triplicate and differences in expression, respect to the case report, were valued by t-Student 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 wild type or mutated allele or both were expressed in the patient parathyroid lesion. Sequence imaging were obtained by ABI Prism DNA Sequencing Analysis Software (Fig. 2C, 2D).

Immunohistochemistry
The specimen was fixed in 10% formalin before being processed in paraffin. Representative sections of the lesions were selected for immunohistochemical analysis. As primary antibodies we used mouse monoclonal anti-p27Kip1 (clone SX53G8, Ventana, Tucson, AZ, USA) and rabbit monoclonal CONFIRM anti-Ki-67 (clone 30-9, Ventana) 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. UltraView Universal DAB Detection Kit (Ventana) was used as the revelation system. Upon completion of the staining run, 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. Negative control was performed by substituting the primary antibody with a non-immune serum at the same concentration. The control sections were treated in parallel with the samples. All sections were dehydrated and mounted with Permount.

**Results**

**Description of the case**

At age 33 the patient underwent a tubaric pregnancy that required salpingectomy. At age 41, she developed symptoms of hypothyroidism due to the Hashimoto thyroiditis. In the same year, primary hyperparathyroidism (PHPT) was diagnosed, with consequent surgical ablation of two enlarged parathyroid glands (left and right inferior; 10x8x4mm and 40x25x20mm, respectively). Both glands were histologically parathyroid adenomas. After surgery, calcium levels normalized, with persistence of PHPT. At age 43 she underwent a hysterectomy for fibroma. At age 48, because of chronic abdominal pain and recurrent obstructive symptoms, adhesiolysis was performed, ileocaecal appendix was removed and incisional hernia was repaired. At age 50, the patient suffered
epigastric symptoms. Esophagogastroduodenoscopy (EGD) showed two small ulcers in the second duodenal portion. Increased plasmatic 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 one across the V-VI hepatic segments. These areas were both diagnosed as neuroendocrine tumours 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). One year after, 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 analogue therapy was restored. At age 50, the patient underwent cervical reoperation to treat the persistent PHPT with removal of an increased right superior parathyroid gland. At age 51, 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 removal of gallbladder. Two large periduodenal nodes were detected and histologically identified as low grade, well-differentiated neuroendocrine tumors (ki67 growth marker less than 2%). No liver metastases were identified during surgery. Treatment with octreotide was maintained, with periodical and specific biochemical and imaging follow-up. Serum CgA did not normalize (43 nmol/L, n.v. 0-6 nmol/L), although SSRS was completely negative. CT and MRI were negative for focal lesions. Gallium 68-labelled positron emission tomography (PET) showed pathological uptake at the level of the right petrous apex. At age 52, the patient complained colic symptoms at the abdominal level with further increase of serum CgA (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. Due to the persistence of the chronic abdominal pain an additional duodeno-pancreatectomy was performed. The histopathological examination showed multiple small (<0.5cm) well differentiated neuroendocrine neoplasias in the duodenal wall, with 1% Ki67, less than 2x50HPF mitoses, and no signs of angioinvasivity or necrosis. Immunohistochemistry was positive for CgA, sinaptophysin and CD56 and negative for gastrin or other pancreatic neuropeptides. The pancreatic parenchyma was free from lesions. Metastasic tissue was present in 1/33 lymph nodes examined.

On 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 25OH vitamin D above 30 ng/ml) and neuroendocrine tumours of the gastro-entero-pancreatic tract, the presence of a MEN1 syndrome was suspected. Biochemical serum exams confirmed the increased values of parathyroid hormone, with ionized calcium in the upper normal range. Secretin stimulation test did not show any pathological increase of the serum levels of entero-pancreatic endocrine peptides. Prolactin levels resulted within the normal range (462-391 mU/L at 0-+30’, n.v. 72-504). MRI showed asymmetry of the pituitary gland (left>right), without focal abnormalities. Pituitary functionality tests were normal [FSH 72.57 U/L. (normal range 26-138), LH 38.34 U/L. (n.v. 16-64), ACTH 17.1 ng/L (n.v. 9-52), GH 0.32 μmol/L (n.v. 0.8-5.4), TSH 2.22 mU/L (n.v. 0.25-3.5), IGF-1 169 ng/ml (n.v. 81-225)]. Lumbar spine (L1-L4) DXA showed osteoporosis with a T-score -2.8 (0.806 g/cm²), with osteopenia at the femoral neck with a T-score -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 which 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 it resulted to be increased in volume (19x9x4mm) with a polylobated shape. The
intraoperative monitoring of PTH showed decrease of PTH to undetectable values (<1.3 pmol/L) 60 minutes from parathyroidectomy of the left superior gland, confirming the removal of all functioning parathyroid tissue 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 of 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 exams for recurrent endocrine tumours 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 RNA later and sent to the laboratory for loss of heterozigosity (LOH) evaluation.

Calcium gluconate IV infusion (2 ampoules, 10%) was required during the first and second post-operative 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 one 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 clinical guidelines. Since 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 one-year follow-up.
Genetic analyses

Genetic analyses in the proband showed lack of MEN1 gene mutations either in the coding region or intron-exon junctions. Large deletion encompassing MEN1 locus on chromosome 11q13.1 were excluded. Mutational analysis of CDKN1B gene on leukocytes 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 found, in heterozygous status, also in the blood-derived genomic DNA from the patient’s 35 years old son. He did has not yet presented 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, PRL=162-122 mU/ml).

Genetic analysis of CDKN1B sequence on somatic DNA from the hyperplastic parathyroid tissue confirmed the presence of the 371delCT mutation (Fig. 2A, 2B). The mutation was found in heterozygous status, indicating the absence of CDKN1B LOH at somatic level, with retention of the wild type copy of CDKN1B in the hyperplastic parathyroid tissue (Fig. 2C, 2D).

CDKN1B mRNA resulted to be expressed in CDKN1B mutated parathyroid hyperplastic tissue even more than in adenomatous CDKN1B non-mutated parathyroid tissue (obtained from a patient with sporadic primary hyperparathyroidism and used as control) with only wild type allele of CDKN1B being expressed, as revealed by DNA sequencing of cDNA (Fig. 3).

CDKN1B mRNA resulted to be expressed in CDKN1B mutated parathyroid hyperplastic tissue (Figure 3). Interestingly, the expression of CDKN1B resulted to be 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 (Figure 4). Moreover only the wild type allele of CDKN1B resulted to be expressed in our patients as revealed by DNA sequencing of cDNA (Fig. 2E, 2F).
On histologic 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 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 \textit{MEN1} oncosuppressor gene \cite{12} and MEN2, caused by germline gain-of-function mutations of \textit{RET} proto-oncogene \cite{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 \textit{CDKN1B} gene \cite{4}. Following this original description, other patients presenting a MEN1 phenotype, but no germline mutations in the \textit{MEN1} gene were described \cite{1,4,10}. In the evaluated cases \textit{CDKN1B} gene mutations generate a decrease of 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 of p27 expression in tumoral tissues or its delocalization from nucleus to cytoplasm \cite{4-6,8}. One of the identified mutations consists in a nucleotide variation at a stop codon with formation of a protein longer than normal, mostly localized in the cytoplasm than in the nucleus, with low stability and reduced CDK2 binding \cite{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 tumour suppressor gene. A mutated transcript can determine a truncated protein or an altered protein mainly localized in the cytoplasm than in the nucleus with lower stability and with a diminished CDK2 binding property [6]. During MEN4 tumorigenesis, the principal mechanism responsible for p27 reduced expression 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]. Differently than in normal conditions, in tumors the p27 protein mainly localized in the cytoplasm is rapidly degraded through ubiquitylation by the KPC ubiquitin ligase and proteasome-mediated degradation [15-17]. In MEN4 syndrome, differently than in MEN1, somatic LOH seems to be a rare event, even though only few MEN4 endocrine tumours have been examined for somatic LOH, which has never been found in the parathyroid gland adenomas, but only in a cervical neuroendocrine metastatic carcinoma and in a bronchial metastatic carcinoid [5,6], suggesting that LOH in MEN4 syndrome could be associated to the aggressive and malignant progression of the neoplasia.

We describe here a case of MEN4 syndrome with various and recurrent endocrine tumors of the parathyroids and gastro-entero-pancreatic tract, associated to a novel CDKN1B mutation, consisting of a heterozygous deletion of CT 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 wild type CDKN1B mRNA allele being expressed at the mRNA level, a significantly higher expression of CDKN1B mRNA and and reduced nuclear p27 staining. Altogether these results suggest that, in this case, the down-regulation of p27 protein expression could be at post-transcriptional and/or post-translational level.

Interestingly, all the MEN4 patients, described to date, showed a relatively late age of clinical expression when compared to MEN1 patients. PHPT has a high penetrance in MEN4 and,
similarly 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 versus 20-25 years in MEN1) [1,4-8]. 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 parathyroid [1, 4-8]. 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 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 tissue.

For its frequency, anterior pituitary adenoma is the second manifestation of MEN4 syndrome. Both functioning (GH or ACTH) and non-functioning tumours have been observed [4-6,8]. The present case showed increase in values of prolactin without relevant morphologic evidence of lesion at the pituitary level.

Only one of the 8 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].

Positive family history was described only in three MEN4 cases and in these cases the relatives, inheriting the \textit{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 resulted to bear the same mutation of his mother, without any clinical manifestation or sign of MEN-4 related endocrine dysfunctions. These findings support the late onset of the disease and will make possible to follow the natural history of this disorder in an asymptomatic carrier.

Interestingly, all the patients affected by MEN4 are women and the great majority of clinical manifestations have aroused around the age of menopause [1, 5] or after [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 the index case, who does not present 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 of 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 breast tumor cells, showing that dihydrotestosterone down-regulates p27 through the ubiquitin-proteasome system by leading to the direct binding of p27 to SKP2 ubiquitin ligase, independently 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 [1,4-8]. This makes difficult to uncover 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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Legends to the Figures

Figure 1. Schematic representation 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: grey symbol).

Figure 2. Sequencing of CDKN1B gene. Panels A and B represent, respectively, the forward and reverse sequences of CDKN1B mutation in genomic DNA from patient’s venous blood sample. Panels C and D represent, respectively, the forward and reverse sequences of CDKN1B mutation in somatic DNA from hyperplastic parathyroid tissue of CDKN1B mutated patient. Sequences evidenced the presence of both mutated and wild type alleles in pathological tissue. Panels E and F represent, respectively, the forward and reverse sequences of CDKN1B mutation in mRNA (reverse transcribed to cDNA) from hyperplastic parathyroid tissue of CDKN1B mutated patient. Sequences evidenced the presence (expression) of only wild type CDKN1B mRNA, and not mutated one, in pathological tissue.

Figure 3. Expression of CDKN1B mRNA in the CDKN1B mutated parathyroid lesion and in a CDKN1B non mutated parathyroid adenoma. Panel A shows the result of CDKN1B mRNA PCR amplifications in CDKN1B mutated parathyroid lesion and CDKN1B non-mutated parathyroid adenoma as control. Panel B shows the graphic representation of the CDKN1B/S18 expression ratio in CDKN1B mutated parathyroid lesion and CDKN1B non-mutated parathyroid adenoma.

Figure 4. Graphical representation of 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 CDKN1 mutations, 3: #1 sporadic parathyroid adenoma negative for MEN1 and CDKN1B mutations; 4: #2 sporadic parathyroid adenoma negative for MEN1 and CDKN1B mutations; 5: MEN1 parathyroid adenoma; 6: CDKN1B positive proband; sporadic parathyroid carcinoma negative for MEN1 and CDKN1B mutations.

* indicates a significant different expression relative to sample 6, evaluated with t-Student test (p<0.01).

Figure 5. Histological phenotype of pathologic parathyroid tissue.

Panel A. Hematoxylin-eosin coloration of the pathologic parathyroid tissue from the proband (original magnification x50). The pathological tissue showed a nodular growth pattern with foci of clear cells. Fat cells were identified as single cells scattered throughout the parenchyma.

Panel B. Immunohistochemistry for p27 protein in pathological parathyroid tissue of the proband (original magnification x100). Nuclear immunoreactivity for p27 was markedly reduced with respect to normal endothelial cells, positive for p27 and serving as internal control (inset).

Panel C. ki-67 coloration of the pathologic parathyroid tissue from the proband (original magnification x100). Nuclear expression of ki-67 positive cells was negligible.
## Table 1: Phenotype and genotype of published MEN4 patients.

<table>
<thead>
<tr>
<th>Author</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/27 gene mutation</th>
<th>Immunostaining for p27 protein Tumor [Staining]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellegata NS et al. Ref. #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>Female GH-secreting pituitary adenoma [30 yrs]</td>
<td>Father with acromegaly (not tested for p27 variant); 2 genetic carriers genders asymptomatic [44 yrs and teenager] 1 positive female with renal angiomyolipoma [55 yrs]</td>
<td>No</td>
<td>Renal angiomyolipoma [No]</td>
</tr>
<tr>
<td>Georgitsi M Ref. #5</td>
<td>Frameshift c.59_77dup19 (6K25) [P27 protein 69 amino acids shorter than wild type 27 and with a complete different amino acid sequence after codon 25]</td>
<td>Female</td>
<td>Small-cell neuroendocrine cervical carcinoma [45 yrs]</td>
<td>First degree relatives free from MEN1-related lesions</td>
<td>No</td>
<td>Cervical neuroendocrine carcinoma [Yes]</td>
</tr>
<tr>
<td>Agarwal SK et al. Ref. #1</td>
<td>ATG-7G&gt;C in the 5'UTR [Reduced expression of p27]</td>
<td>Female</td>
<td>1°HPT (one parathyroid gland) [61 yrs]</td>
<td>2 asymptomatic daughters genetic carrier [47 yrs and 48 yrs]</td>
<td>No</td>
<td>One parathyroid gland adenoma [No]</td>
</tr>
<tr>
<td>Agarwal SK et al. Ref. #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 (2 affected parathyroid glands) [50yrs]</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Agarwal SK et al. Ref. #1</td>
<td>STOP-&gt;199Q (c.595T&gt;C) [P27 longer of 60 amino acids than wild type and reduced expression of p27]</td>
<td>Female</td>
<td>ZES and masses in duodenum and tail of pancreas [50 yrs]</td>
<td>Monozygotic twin sister with PHPT [66 yrs] Two not genetically tested relatives with PHP: Aunt [52 yrs] Female cousin [NA]</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Molatore S et al.</td>
<td>P69L (c.678C&gt;T) [Bilateral multiple]</td>
<td>Female</td>
<td>Bronchial carcinoid</td>
<td>NA</td>
<td>NA</td>
<td>Parathyroid adenoma</td>
</tr>
<tr>
<td>Authors</td>
<td>GAGA Deletion in 5’UTR</td>
<td>Female</td>
<td>Gastric carcinoid [69 yrs]</td>
<td>PHPT [74 yrs]</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Male Ga et al.</td>
<td>GAGA deletion in 5’UTR (c.-32_29delGAGA)</td>
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</tr>
</tbody>
</table>

Footnotes: PHPT: primary hyperparathyroidism; NA: not available; ORF: open reading frame
1 = CDKN1B mutated parathyroid lesion
2 = CDKN1B non-mutated parathyroid adenoma

A

B

CDKN1B expression (ImageJ software evaluation)

169x117mm (72 x 72 DPI)
t-Student

* = p<0.001

Relative quantity (AU)

samples

1 2 3 4 5 6 7

174x131mm (72 x 72 DPI)