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# Multiple Endocrine Neoplasia Type 4

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**Abstract**

A few years ago a novel multiple endocrine neoplasia syndrome, named multiple endocrine neoplasia type 4 (MEN4), was discovered thanks to studies conducted on a MEN syndrome in the rat (named MENX). The rat and the human syndromes are both caused by germline mutations in the *Cdkn1b/CDKN1B* gene, respectively. This gene encodes p27Kip1, a putative tumor suppressor which binds to and inhibits cyclin/cyclin-dependent kinase complexes, thereby preventing cell cycle progression. MEN4 patients carry heterozygous mutations at various residues of p27Kip1 and present with endocrine lesions mainly belonging to a MEN1-like spectrum: their most common phenotypic features are parathyroid and pituitary adenomas. Recently, germline mutations in p27kip1 were also identified in patients with a sporadic parathyroid disease presentation. In vitro functional analysis of several *CDKN1B* sequence changes identified in MEN4 patients detected impaired activity of the encoded p27Kip1 variant proteins (e.g. reduced expression, mislocalization or poor binding to interaction partners), thereby highlighting the characteristics of the protein which are critical for tumor suppression. Although the number of MEN4 patients is low, the discovery of this syndrome has demonstrated a novel role for *CDKN1B* as a tumor susceptibility gene for neuroendocrine tumors. Here, we review the clinical characteristics of the MEN4 syndrome and the molecular phenotype of the associated p27Kip1 mutations.


Multiple endocrine neoplasia type 4 (MEN4) is the latest member to join the family of MEN syndromes. These syndromes are autosomal dominant disorders characterized by the occurrence of tumors involving two or more endocrine glands, although tumors can also develop in nonendocrine organs. MEN type 1 (MEN1) and type 2 (MEN2) syndromes are the 'classical' MEN syndromes, whose clinical characteristics, tumor spectrum and molecular genetics have been elucidated. MEN1 is characterized by tumors of the parathyroid, endocrine pancreas and anterior pituitary and is caused by loss-of-function mutations of the *MEN1* gene [1, 2]. MEN2 is a syndrome of medullary thyroid carcinoma (MTC) occurring in association with pheochromocytoma and multigland parathyroid adenoma. Three variants are recognized: MEN2a, the most common, characterized by all three above-mentioned tumors; MEN2b,

where parathyroid is not involved and pheochromocytoma develops together with marfanois habitus, gastrointestinal disorders and multiple mucosal ganglioneuromas; FMTC, where MTC is the only manifestation. MEN2 is caused by activating mutations of the *RET* proto-oncogene [1, 2].

While *RET* mutations virtually explain all MEN2 cases, in approximately one third of the clinically suspected MEN1 patients *MEN1* mutations are not found. While this might be due to the mutation lying outside the regions tested, it also suggests that other predisposing genes may play a role in causing this phenotype. Our recent work on a rat strain that spontaneously developed a MEN-like phenotype (named MENX syndrome) demonstrated that a germline mutation in the *Cdkn1b* gene, encoding the cell cycle inhibitor p27Kip1 (hereafter p27), is responsible for this syndrome [3]. Thus, we decided to check whether mutations in the human homologue *CDKN1B* could explain some of the MEN1-like cases without mutations in *MEN1*. We screened for the presence of *CDKN1B* germline mutations, several patients fulfilling the above criteria and we identified a germline heterozygous nonsense mutation at codon 76 in a female proband with pituitary adenoma and primary hyperparathyroidism. This mutation segregated with the predisposition to a MEN1-like phenotype in the proband's family [3]. Subsequently, we and other groups have identified additional index cases presenting with a MEN1-like phenotype and carrying heterozygous *CDKN1B* gene mutations. Altogether, these findings identified *CDKN1B* as a novel tumor susceptibility gene for multiple endocrine neoplasia and led to the recognition of a novel MEN syndrome named MEN4 caused by mutations in p27 (OMIM No. 610755). Recently, *CDKN1B* mutations were also identified in patients with a sporadic presentation of endocrine tumors [4]. The overall number of MEN4 patients is still too low to allow us to formulate guidelines for the clinical diagnosis of the disease or for patient management. However, since new mutation-positive cases keep being discovered, *CDKN1B* needs to be considered as the predisposing gene in cases presenting with multiple endocrine tumors and no mutations in other tumor susceptibility genes.

In this chapter, we summarize the clinical features of the MEN4 cases described so far and we compare them with other familial endocrine tumors syndromes (table 1). We also present the functional characterization of the associated germline *CDKN1B* mutations in an attempt to better understand the role of p27 in the susceptibility to neuroendocrine tumors.

## Phenotypic Features of Patients Carrying *CDKN1B* Germline Mutations

### *Primary Hyperparathyroidism*

To date, 12 index cases having germline nucleotide substitutions in *CDKN1B* have been reported in the literature. The phenotypic features of these patients are summarized in table 2. The most common feature is primary hyperparathyroidism (PHPT), which was observed in 10 of the 12 patients (incidence 81%). In the majority of the



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**Table 1.** Tumor spectrum of the various MEN syndromes; the main clinical features of MEN1, MEN2A, MEN2B and MEN4 are tabulated

| | MEN1 | MEN2A | MEN2B | MEN4 |
|---|---|---|---|---|
| Pituitary adenoma | + | | | + |
| Parathyroid adenoma | + | + | | + |
| Medullary thyroid tumors | | + | + | |
| Pheochromocytoma | | + | + | |
| Paragangliomas | | + | | |
| Insulinoma | + | | | |
| Neuroma | | | + | |

**Table 2.** Germline changes in the *CDKN1B* gene and the clinical characteristics of the mutation-positive patients

| *CDKN1B* mutation | Parathyroid adenoma | Pituitary tumor | Other manifestations |
|---|---|---|---|
| *MEN4 (MEN1-like)* | | | |
| ATG-32–29del | PHPT | | gastric carcinoid tumor |
| ATG-7G>C | PHPT | | bilateral adrenal mass non-functioning<br>uterine fibroid |
| K25fs | PHPT | ACTH-secreting | |
| A55T | PHPT (hepatic metastasis) | | Zollinger-Ellison syndrome<br>gastrinoma |
| P69L | PHPT | nonfunctioning | bronchial carcinoids, papillary thyroid carcinoma,<br>multiple lung metastases |
| W76X | PHPT | GH-secreting (acromegaly) | |
| P95S | PHPT (2 parathyroid tumors) | | Zollinger-Ellison syndrome<br>mass in duodenum and tail of pancreas |
| Stop>Q | PHPT (3 parathyroid tumors) | | |
| *MEN4 (others)* | | | |
| G9R | PHPT | | |
| K96Q | | PRL-secreting | breast tumor |
| I119T | | GH-secreting | |
| P133T | PHPT (1 parathyroid tumors) | | |

Mutations are numbered with reference to the cDNA sequence AY890407 (GenBank).
ACTH = Adrenocorticotropic hormone; GH = growth hormone; PHPT = primary hyperparathyrodism; PRL = prolactin.



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**Table 3.** Genes predisposing to hereditary PHPT and pituitary adenoma

| Gene | Tumor type |
|---|---|
| **PHPT** | |
| *CaSR* | parathyroid hyperplasia/adenoma |
| *CDKN1B* | parathyroid hyperplasia/adenoma |
| *HRPT2* | parathyroid adenoma/carcinoma |
| *MEN1* | parathyroid hyperplasia/adenoma |
| *RET* | parathyroid hyperplasia/adenoma |
| **Pituitary Adenoma** | |
| *AIP* | GH-secreting |
| | GH/PRL-secreting |
| *CDKN1B* | PRL-secreting |
| | GH-secreting |
| | ACTH-secreting |
| | nonfunctioning |
| *MEN1* | PRL-secreting |
| | GH-secreting |
| | GH/PRL-secreting |
| | TSH-secreting |
| | ACTH-secreting |
| *PRKAR1* | GH-secreting |

ACTH = Adrenocorticotropic hormone; GH = growth hormone; PRL = prolactin; TSH = thyroid-stimulating hormone.

mutation-positive patients the presence of a parathyroid tumor as the underlying cause of PHPT was confirmed by surgical resection. The age of onset was quite variable, ranging from 46 to 74 years of age.

PHPT is the most common feature of the MEN1 syndrome too, occurring in more than 90% of patients [5]. In addition to *CDKN1B* mutations, nucleotide substitutions in *RET*, *CaSR* (calcium-sensing receptor) and *HRPT2* (parafibromin) genes are known to predispose to PHPT (table 3) [6]. Biochemical analysis of these patients usually detects hypercalcemia, which is associated with increased circulating levels of parathyroid hormone. Currently there is no medical treatment for PHPT and surgical removal of the affected parathyroid gland(s) is the only available option for a definitive treatment.

*Pituitary Adenoma*

The second most common tumor type occurring in *CDKN1B* mutation carriers is pituitary adenoma, observed in 5 of 12 (41.6%) index cases. Pituitary adenomas are common tumors of the anterior lobe of the pituitary gland and account for about 15% of intracranial neoplasm. These tumors are usually classified based on the hormone secreted by the tumor cells or because these cells do not produce any hormone (nonfunctioning tumors) [7]. Thus, there are lactotroph adenomas which secrete prolactin



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(PRL), somatotroph adenomas producing growth hormone (GH), corticotroph adenomas secreting adrenocorticotropic hormone (ACTH), and thyrotroph adenomas secreting thyroid-stimulating hormone (TSH). Gonadotroph adenomas may secrete follicle-stimulating hormone (FSH) and/or luteinizing hormone (LH), but many of them are not associated with hormone excess and are therefore nonfunctioning tumors. The clinical manifestations of pituitary tumors depend upon the secreted hormone or the size of the tumor in the case of nonfunctioning adenomas. MEN4 patients develop various types of pituitary adenomas, as summarized in table 2. Two patients developed a GH-secreting tumor and had features of acromegaly, 1 patient with ACTH-secreting tumor had Cushing's disease. One patient had a nonfunctioning tumor and another a suspected prolactinoma (elevated prolactin levels in blood). Age of onset was sometimes earlier than typical sporadic cases, although highly variable (from 30 to 79 years).

Pituitary adenomas are a component tumor feature of MEN1 and of other inherited tumor syndromes, such as Carney complex and familial isolated pituitary adenoma (FIPA). The vast majority of MEN1-associated pituitary adenomas are prolactinomas (60% of cases), followed by GH-secreting tumors (25%). Mutations in the protein kinase A regulatory subunit-1-alpha *(PRKAR1A)* gene cause Carney complex, a familial multiple neoplasia syndrome having multifocal hyperplasia of the somato-mammotropic pituitary cells as a component feature [8]. More recently, the aryl hydrocarbon receptor interacting protein (AIP) was identified as a pituitary adenoma predisposing gene. *AIP* gene mutations explain up to 30% of FIPA families. FIPA-associated pituitary adenomas are usually GH- or GH/PRL-secreting adenomas, with few prolactinomas or other tumor subtypes (table 3) [9]. Being that the number of mutation carriers identified so far is small, it is not possible to ascertain whether *CDKN1B* variants do indeed equally affect the various pituitary cell lineages or they preferentially target a specific one. MEN1-associated pituitary tumors tend to be more aggressive than their sporadic counterpart, usually respond poorly to conventional therapy and tend to recur [10]. We do not yet know whether the pituitary adenomas developing in the context of *CDKN1B* mutations are also more aggressive. Interestingly, the GH-secreting adenoma associated to the p27W76X mutation (table 2) showed sella and cavernous sinus invasion, and, at histopathological examination, displayed cell atypia and a quite elevated mitotic activity, suggesting aggressive tumor behavior [3].

Although usually benign, pituitary adenomas may cause severe morbidity secondary to excessive hormone production or, in nonfunctioning adenomas, due to local invasion of adjacent neuroanatomical structures. Transsphenoidal surgery is currently the treatment of choice for pituitary adenomas, but it is not always curative. If residual tumor is discovered or if the tumor recurs, radiation therapy is performed, but this approach is associated with serious long-term complications [11]. Receptor-mediated pharmacological therapy is also used but the response varies greatly among patients and tumor subtypes [12].



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*Associated Tumors*

MEN4 patients may develop tumors involving organs other than parathyroid and pituitary glands. Indeed, neuroendocrine carcinomas of the cervix, carcinoids in the bronchi and stomach, and a gastrinoma have also been observed (table 2). Two MEN4 patients presented with masses in the pancreas or in the adrenal glands, but the precise nature of these lesions is currently unknown. Interestingly, endocrine pancreatic tumors and adrenal tumors are also observed in MEN1 patients, and so are lung carcinoids, which in MEN1 patients occur with a frequency of about 36% [13].

## The *CDKN1B* Tumor Susceptibility Gene

The identification of *CDKN1B* as a tumor susceptibility gene for multiple endocrine tumors originated from studies of MENX, a MEN-like syndrome in the rat. This multitumor syndrome was discovered about 10 years ago when a Sprague-Dawley-derived rat colony spontaneously started to develop multiple neuroendocrine tumors at a young age, including anterior pituitary adenoma, adrenal and extra-adrenal pheochromocytoma, thyroid C-cell hyperplasia, and parathyroid hyperplasia [14]. The MENX syndrome was reported to be inherited as a recessive trait. Linkage studies finally mapped the *MENX* locus to a 3-Mb region containing several genes, including *Cdkn1b*, encoding the cell cycle inhibitor p27. This gene was selected as a putative candidate gene for the rat syndrome because p27 knockout mice develop pituitary gland tumors [15], an organ also affected by the MENX syndrome, and p27 is a putative tumor suppressor, which is consistent with the recessive mode of inheritance observed in MENX rats. Upon sequencing of the *Cdkn1b* gene in affected and unaffected littermates, a tandem duplication of eight nucleotides in exon 2 (c.520–528dupTTCAGAC; RefSeq: NM_031762.3), which causes a frameshift, was identified in all the affected rats. The mutated allele encodes a protein having a C-terminal sequence different from that of the wild-type protein, which was later found to be highly unstable and therefore absent, or present at very low level, in the tissues of affected rats [3]. Capitalizing on this finding, we then sequenced *CDKN1B* in patients with a MEN1-like phenotype and no MEN1 gene mutations and we identified the first germline mutation in a patient with PHPT and pituitary adenoma.

The *CDKN1B* gene has two coding exons resulting in a 2.5-kb-long coding region. *CDKN1B* is located on chromosome 12p13, a chromosomal region known to undergo hemizygous loss in hematological malignancies. The MEN4-associated germline mutations identified in the coding sequence of *CDKN1B* are for the vast majority missense mutations (8/12) although also a nonsense mutation and an insertion (duplication) were detected (fig. 1). Two nucleotide changes occur in the 5′ untranslated region of the transcript (one missense and one deletion). Each index case presents with a distinct germline mutation. The p27 variants which were characterized at the func-



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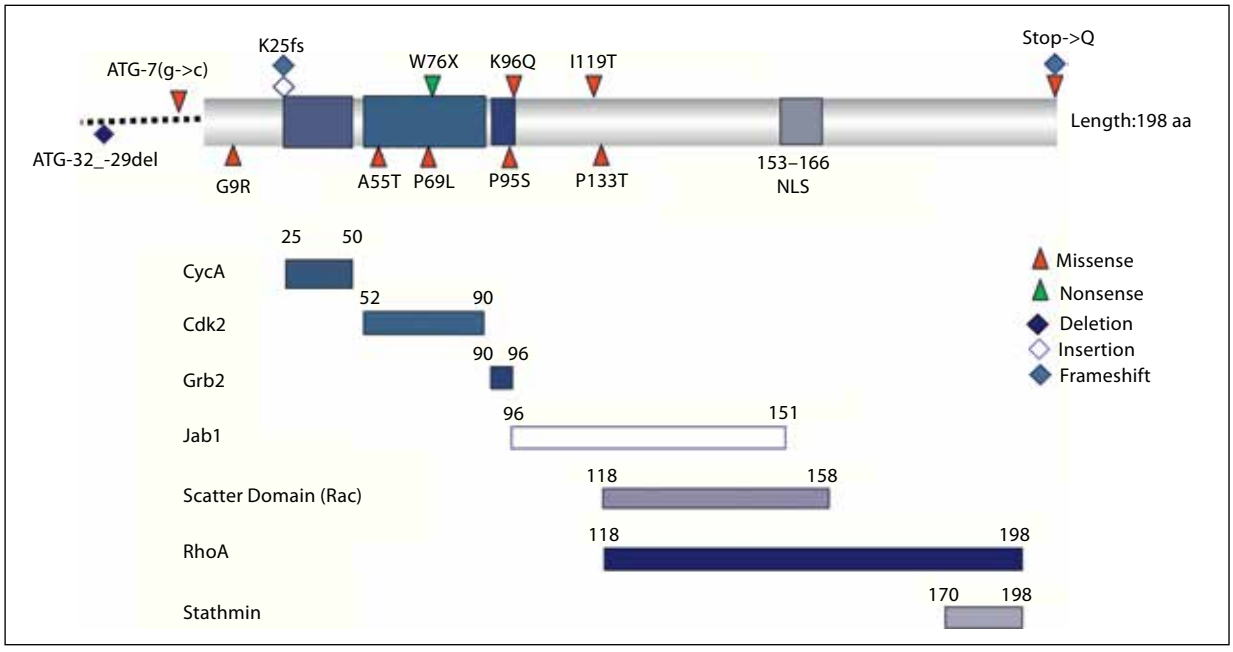


**Fig. 1.** Schematic structure of the p27 protein. The p27 protein is composed of several protein-binding domains and contains a nuclear localization signal (NLS). The position of the germline mutations is indicated by colored symbols. CycA = Cyclin A; Cdk2 = cyclin-dependent kinase 2; Grb2 = growth factor receptor-bound protein 2; Jab1 = c-jun activation domain binding protein 1; RhoA = Ras homolog gene family, member A.

tional level seem to be inactivating, and this is consistent with the tumor suppressive role of the protein (see below).

Somatic *CDKN1B* mutations are extremely rare in human tumors and to date only a handful has been reported in the literature, namely: a nonsense mutation in an adult T-cell leukemia/lymphoma (p27W76X) [16]; a nonsense mutation in a breast cancer sample (p27Q104X) [17]; a missense change in an unclassified myeloproliferative disorder (p27I119T) [18]. Lately, a somatic missense change (p27P133T) and a 25 nucleotide deletion starting at codon 25 (c.582del25) in *CDKN1B* were identified in sporadic parathyroid adenomas [4]. The tumor tissue carrying the c.582del25 change showed loss of heterozygosity of the wild-type allele and no immunoreactivity for p27. Interestingly, 3 of 4 of these somatic changes (p27W76X, p27I119T, p27P133T) were also identified in the germline of individuals with neuroendocrine tumors. Since the p27 protein is so rarely mutated, the finding that the same mutation arose independently in somatic and germline settings seems to suggest that these variant proteins have a pro-oncogenic effect.

Several single nucleotide polymorphisms (SNPs) have been identified in the human *CDKN1B* gene (http://www.ncbi.nlm.nih.gov/snp), including three which are poten-



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tially functional: –838C>A (rs36228499), –79C>T (rs34330) and 326T>G (V109G, rs2066827). Several studies have demonstrated a significant association between coding SNP rs2066827 (V109G) and a variety of cancers including those of the prostate, ovary, breast and, recently, also medullary thyroid carcinoma [19]. Whether this amino acid substitution affects p27 levels or function is currently unknown. Recently, SNP –79C>T (rs34330) was associated to the risk of developing papillary thyroid carcinoma (follicular variant) [20]. The authors went on to demonstrate that the variant allele associates with reduced gene transcription and, consequently, with lower p27 protein levels.

### *CDKN1B* Germline Mutations in Patients with a MEN1-Like Phenotype (MEN4)

To date, 8 germline base substitutions in *CDKN1B* have been identified in association with a MEN1-like phenotype. Most of them were missense mutations, thus it is intrinsically difficult to assign these variants a pathological role. However, several of them are usually not found in control unaffected individuals and they affect the protein's function (see below), thus they have been classified as potentially pathogenic. We refer to them as 'mutations' throughout the text. The changes with no detectable molecular phenotype and/or observed in control individuals are considered genetic variants of unknown significance. Since for most index cases only a few first-degree relatives were available for genetic analysis, if at all, segregation with the disease phenotype in these families was not used as the main criterion for assigning a possible pathogenic role to *CDKN1B* variants. All the identified changes occur in heterozygosity, thereby suggesting a dominant inheritance of the tumor predisposition. We here present these changes following their position along the gene sequence (from 5′ to 3′).

Two mutations have been found in the 5′ UTR region of the *CDKN1B* gene. One of them is a heterozygous GAGA deletion at the –29 position (c.–32/–29del; RefSeq: AY890407), which was identified in a Spanish female patient with gastric carcinoid tumor (at age 69) and PHPT (at age 74). In peripheral blood leukocytes from this patient, the *CDKN1B* mRNA was significantly reduced compare to a normal healthy control [21]. In a study of American patients with a MEN1-like phenotype and no MEN1 gene mutations, Agarwal et al. [22] discovered a mutation at the –7 position in the Kozak sequence (ATG–7G>C) in a patient with a parathyroid tumor, bilateral adrenal masses and uterine fibroids. No loss of heterozygosity was found in the tumor but protein expression could not be assessed.

The remaining 6 mutations occur in the coding sequence of the *CDKN1B* gene. A 19-bp duplication (c.59–77dup19) in exon 1, which causes a frameshift after codon 25, was identified in a Dutch patient diagnosed with three lesions compatible with MEN1: ACTH-secreting pituitary adenoma (Cushing's disease; age 46 years), small-cell neuroendocrine cervical carcinoma (age 45 years), and hyperparathyroidism (age 47 years). The resulting variant transcript is predicted to encode a p27 protein having an amino acid sequence different from that of the wild-type protein after the site of the



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insertion, and being 69 amino acids shorter (p.K25fs). The cervical carcinoma of the patient showed loss of the wild-type *CDKN1B* allele and no p27 protein expression, while the other tumor tissues could not be analyzed [23].

Recently, a cohort of familial and sporadic Spanish patients with a MEN1 phenotype was screened for mutations in *MEN1*, *CDKN1B* and *AIP*, and a novel missense mutation in *CDKN1B* (c.163G>A; p. Ala55Thr, A55T) was found in a lady presenting with Zollinger-Ellison syndrome with gastrinoma and hepatic metastases (at age 42 years) and PHPT (at age 51 years) [24].

The screening of a series of Italian patients with suspected MEN1 identified a *CDKN1B* germline mutation in a 79-year-old Caucasian female patient diagnosed with PHPT caused by a parathyroid adenoma (at age 67 years) and a papillary thyroid carcinoma (at age 64 years). Magnetic resonance imaging (MRI) revealed a nonfunctioning pituitary microadenoma in this lady (at age 79 years). She also developed bilateral multiple lung metastases of a bronchial carcinoid and type 2 diabetes mellitus. This index case carries a missense mutation at codon 69 of p27 which results in a proline to threonine amino acid substitution (c.678C>T, p.P69L) [25].

The heterozygous TGG>TAG (c.227G>A) nonsense mutation at codon 76, which determines the premature truncation of the protein (p.W76X), was the first *CDKN1B* mutation to be detected in a 48-year-old Caucasian female presenting with GH-secreting pituitary tumor (acromegaly; at age 30 years) and PHPT (at age 46 years). In the family of the index case, this mutation is inherited together with the predisposition to MEN1-like tumors. Indeed, a mutation-carrier sister of the proband presented with renal angiomyolipoma (at age 55 years), a MEN1-associated tumor [3]. The normal renal tissue of this woman showed cytoplasmic immunoreactivity for p27, while her angiomyolipoma had no p27 staining [25].

An individual affected by PHPT and displaying masses in both duodenum and pancreas was found to carry a CCC>TCC missense mutation at codon 95, leading to a proline to serine amino acid change (P95S). Another sequence variant was found in a patient with a family history of PHPT. This change transforms the stop codon in glutamine (TAA>CAA; stop>Q), thereby leading to a protein predicted to be 60 amino acids longer than the wild-type one [22].

The analysis of family members of the mutation-positive index cases (when possible) showed that some *CDKN1B* sequence changes are also found in a few of their nonsymptomatic first-degree relatives, suggesting that some mutated alleles may have incomplete penetrance.

## *CDKN1B* Germline Mutations in Endocrine Disease

In addition to patients with a MEN1-like phenotype, also patients with a FIPA phenotype and no mutations in the *AIP* gene were found to occasionally carry germline *CDKN1B* gene variants. In 88 AIP mutation-negative FIPA families (for a total of 124 af-



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fected subjects) the *CDKN1B* gene was sequenced and two point mutations were found in two patients: c.286A>C (p.K96Q) and c.356T>C (p.I119T) (table 2; fig. 1). These sequence changes led to functional or conformational alterations in the encoded p27 proteins in vitro (see below). Additional variants were identified in this FIPA patients cohort (p.S56T, p.T142T, c.605 + 36C>T) but are likely to be nonpathogenic because they were occasionally observed in control unaffected individuals and were not predicted to affect p27 function by in silico modeling [26].

So far, we presented *CDKN1B* mutations occurring in patients selected because of a familial presentation of neuroendocrine disease, either MEN1-like or FIPA-like. However, it was recently demonstrated that germline p27 changes can be identified also in patients with an apparently sporadic presentation of neuroendocrine tumors. Indeed, we discovered two novel germline *CDKN1B* variants in a study of 90 single gland parathyroid adenomas, presumed to be sporadic [4]. Specifically, one patient carried a heterozygous germline single nucleotide change at base 25 in *CDKN1B* exon 1 (c.25G>A), which would result in a glycine to arginine substitution at codon 9 (G9R) of the translated p27 protein product. This patient, a 68-year-old man, had a typical single-gland, mildly symptomatic tumor presentation. The germline DNA of another patient, a 53-year-old woman presenting with fatigue, slightly elevated serum calcium and parathyroid hormone levels, carried a heterozygous single nucleotide substitution (c.397C>A), directing a proline to threonine substitution at position 133 (P133T) of the encoded p27 protein [4].

## Function of the p27 Protein

To fully appreciate the effect of the different *CDKN1B* mutations on the function of the encoded variant p27 proteins, it is important to briefly summarize the most relevant functions of wild-type p27. It is a ubiquitously expressed protein, member of the KIP1 family of cyclin-dependent kinase (CDK) inhibitors [27], together with p21 and p57. The wild-type p27 protein is 198 amino acids long and possesses a nuclear localization signal (NLS) between amino acids 153 and 166, which targets the protein to the cell nucleus (fig. 1). The most important function of p27 is to regulate the progression from the G1 to the S phase of the cell cycle by inactivating the cyclin A,E/ CDK2 complexes. The substrate of CDK2 is the retinoblastoma protein (pRb). Upon phosphorylation by CDK2, pRb releases members of the E2F family of transcription factors which in turn activate the transcription of genes required for the progression into the S phase. Thus, in the presence of p27, cell cycle progression is blocked, and this is an important mechanism regulating tissue homeostasis in most adult organs [28]. Due to the important role of p27 in cell growth, its function is tightly regulated through different mechanisms modulating the intracellular level of the protein, its localization or posttranslational modifications. The best known mechanism regulating the amount of p27 is ubiquitin-mediated proteasomal degradation of the protein



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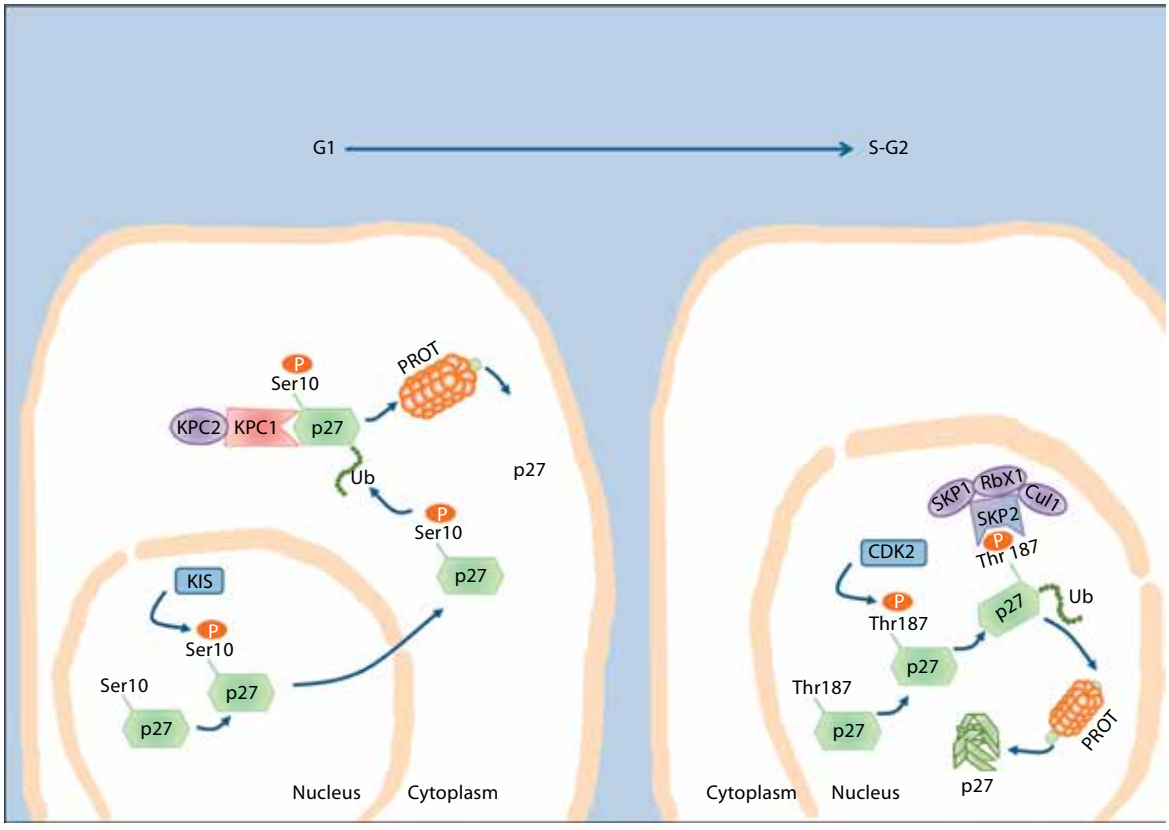


**Fig. 2.** Diagram illustrating the known p27 degradation pathways. After the dissociation of p27 from the cyclin E/Cdk2 complex in early G1, a portion of p27 is phosphorylated on Ser10 by kinase-interacting stathmin (KIS) and then exported to the cytoplasm. Once in the cytoplasm, p27 interacts with the KPC1/KPC2 complex that promotes p27 ubiquitylation and degradation of the protein by the proteasome (PROT). Upon mitogenic stimulation, p27 becomes a substrate of the Cdk2 complex which phosphorylates the protein at the Thr187 residue. Phosphorylation at Thr187 allows the interaction with Skp2 and the subsequent ubiquitylation-mediated degradation by the proteasome.

[29]. Two main pathways involved in p27 degradation have been identified (fig. 2). At the beginning of the G1 phase, after the dissociation from the cyclinE-CDK2 complex, p27 is phosporylated at serine 10 (Ser10), the major phosphorylation residue of p27. Phosphorylation at Ser10 increases the binding affinity of p27 to CRM1 (or exportin 1) via the nuclear export signal of p27. This association promotes the export of p27 to the cytoplasm, where it is then ubiquitylated and degraded by the ubiquitylation-promoting complex KPC1 [30].

The second mechanism involved in p27 degradation occurs in late G1 and requires the phosphorylation of the protein at the threonine (Thr) 187 residue by CDK2, which creates a recognition site for the nuclear SKP2 ubiquitin ligase. SKP2 induces polyubiquitylation of p27 and its subsequent degradation by the proteasome (fig. 2) [31].



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The subcellular localization of p27 indirectly regulates the protein's activity by rendering the protein available, or unavailable, to various interacting partners (such as cyclin/CDK complexes) or to degradation pathways. In addition, recent evidence suggests that p27 exerts different functions while in the nucleus or in the cytoplasm, so that the subcellular localization of the protein may also directly affect its function. For instance, while in the nucleus p27 inhibits cyclin/CDK complexes, in the cytoplasm p27 is a necessary assembly factor for cyclin D/CDK4,6 complexes, which in turn promote cell proliferation. Recently, based on genetic studies on mutant mouse strains, it has been suggested that cytoplasmic p27 may have oncogenic properties (see below) [32].

## p27 and Tumorigenesis

*CDKN1B* has been considered an atypical tumor suppressor gene for many years because it is not mutated (or extremely rarely) in human cancers. In contrast, the level of expression of the encoded p27 protein is reduced in the vast majority of human neoplasms. Downregulation of p27 associates with poor clinical outcome in several malignancies, including cancers of the breast, colon, prostate, and ovary, among others [33]. Several studies in various tumor entities demonstrated that *CDKN1B* mRNA levels are similar among samples with high or low p27 protein levels. Therefore, it is believed that the decrease of p27 expression in human tumors is mainly due to post-transcriptional or posttranslational mechanisms. Indeed, in tumors such as colorectal carcinoma and lymphomas, it could be shown that enhanced proteasome-mediated degradation of p27 is responsible for p27 downregulation.

Animal models have significantly contributed to our understanding of the role of p27 in tumorigenesis. p27 knockout mice were generated and analyzed: they develop normally, are usually bigger than their wild-type littermates and show pituitary adenoma of the intermediate lobe as sole tumor phenotype (frequency 100%) [15]. These observations further supported the tumor suppressive role of p27 in tumor formation. Our work on the MENX syndrome demonstrated that a homozygous *Cdkn1b* mutation, causing extreme reduction of the p27 protein levels [34], renders rats susceptible to multiple neuroendocrine tumors formation. Interestingly, heterozygous knockout mice, upon γ-irradiation or treatment with carcinogens, develop tumors at higher frequency and multiplicity compared with their wild-type littermates, indicating that the loss of one *Cdkn1b* allele already predisposes mice to tumor formation [35]. In conclusion, it seems that the amount of p27 protein plays an important role in promoting tumor formation in both humans and rodents.

Recently, many studies have demonstrated a relationship between the mislocalization of p27 and tumorigensis. Indeed, cytoplasmic immunoreactivity for p27 has been observed in several tumor types, including breast cancer and clear renal cell carcinoma, and it is usually associated with high tumor grades and poorer patient sur-



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vival. Sequestration of p27 in the cytoplasm impairs the protein's ability to inhibit cyclin/CDK complexes. Mice homozygous for a mutant p27 protein unable to bind to cyclin/CDK complexes (p27 CK–/CK–) show increased tumor multiplicity (lung, retina, pituitary, ovary, adrenals, spleen, and lymphomas) and aggressiveness when compared with homozygous p27 knockout mice p27 (p27–/–), suggesting that p27CK- plays a pro-oncogenic role. Interestingly, in p27CK–/CK– mice the tissues susceptible to tumor formation abundantly express p27 in the cytoplasm not only in the nucleus, thereby leading to the speculation that the oncogenic role of protein may depend on its cytoplasmic localization [32].

## Functional Effects of p27 Mutations

Most of the *CDKN1B* germline mutations identified in patients have been cloned and studied in vitro to try and elucidate how they associate with tumor predisposition. Only the K25fs and the most recent A55T change have yet to be studied in transfection experiments. The most common molecular phenotypes associated with these allelic variants are a reduced amount of the *CDKN1B* transcript or of the encoded p27 protein. These features are in agreement with the tumor suppressive role of p27 in tumor formation.

U-rich elements in the 5′-UTR region of *CDKN1B* regulate mRNA stability and p27 translation. In vitro transcription/translation assays performed using the c-32_–29del mutation in 5′-UTR region of the gene showed a decreased rate of transcription of the variant transcript and a reduced level of the encoded p27 protein [21]. In contrast to the c-32_–29del mutation, cells transfected with the ATG-7 variant showed a reduction of the expression level of the encoded p27 protein, but no changes at the mRNA level [22].

Concerning the p27G9R mutation, glycine at position 9 in p27 protein is highly conserved among vertebrates, and this amino acid substitution mimics a phosphorylation event on the adjacent Ser10 residue, the most important phosphorylation site of p27. The G9R change makes the protein less stable then wild-type p27, which may explain why that parathyroid tumor tissue of the mutation-positive patient has a low level of expression of p27 [4].

Proline at position 69 of p27 is located in the CDK2 binding domain of the protein (amino acids 52–93) (fig. 1) and we could demonstrate that the mutant p27P69L protein does not bind to CDK2 in vitro. In addition, the P69L change made the protein more unstable than wild-type p27. Our observation that the overexpression of p27P69L associates with reduced growth suppression when compared to expression of wild-type p27 is likely explained by the lower expression level of the mutant protein and by its inability to bind to CDK2 [25].

In contrast to the other mutations so far described, the truncated p27W76X mutant protein lacks the nuclear localization signal and indeed is localized in the cyto-



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plasm in vitro, in the normal tissues of a mutation-positive patient [25]. The inability of p27W76X to enter the nucleus, the cell compartment where p27 exerts its function as a cell cycle inhibitor, abolishes its ability to inhibit the growth of GH3 cells both by clonogenic assay and by stable expression of this variant p27 protein.

The missense mutations p27P95S and p27K96Q are situated in the proline rich domain (amino acids 90–96) of p27 which mediates the binding to the adaptor molecule Grb2, which in turn recruits and leads to the activation of Ras. Both mutant p27 proteins fail to bind to Grb2 in vitro, and this is assumed to affect the ability of p27 to impair the activation of the Ras pathway [22].

The p27I119T protein displays an abnormal migration pattern by polyacrylamide gel electrophoresis, in that it migrates slower than the wild-type protein [26]. The unique migration pattern of p27I119T, suggestive of noncanonical posttranslational modifications, is not due to increased phosphorylation at the newly created threonine residue. Since glycosylation occurs at serine, threonine or aspartic acid residues, the migration shift associated with the 119T residue could be caused by glycosylation of the protein, thereby conferring greater stability. In agreement with this finding, p27I119T is more stable than wild-type p27 in vitro [26].

The p27P133T variant, observed both as germline and somatic change in patients with PHPT, was analyzed in vitro using various assays but no differences in behavior when compared with the wild-type p27 protein could be identified, so this is a change of unknown pathogenic significance [4].

In one patient, a missense mutation changed the stop codon of the protein into Glutamine (p27stop>Q), and this lead to a frameshift and to an encoded protein which is 60 amino acids longer than wild-type p27. When this variant was analyzed in vitro, the p27stop>Q protein was expressed at lower level than wild-type p27 and treatment with a proteasome inhibitor drug rescued protein levels. Thus, it seems that this mutation makes the p27 protein more unstable [22].

## Conclusions

Mutations in *CDKN1B,* although rare, are responsible for a subset of patients with various combinations of endocrine tumors and with no mutations in other susceptibility genes. The most common feature of MEN4 is PHPT followed by pituitary adenomas, most resembling MEN1. However, single endocrine tumors, such as pituitary or parathyroid adenomas, may also be associated with germline *CDKN1B* mutations. The changes so far studied at the functional level reduce the final amount of *CDKN1B* transcript and/or p27 protein, alter the protein's intracellular localization or its ability to interact with partner proteins such as CDK2 or Grb2. Whether complete inactivation of p27 function is required to promote tumor formation in patients is still not clear as only some tumor tissues of mutation-positive patients were examined and some of them show loss of p27 expression while others only have reduced expression.



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Clinical follow-up and genetic analysis of the extended families of p27 mutation-positive probands might help us assess the role of *CDKN1B* genotyping in individualized risk assessment and management of MEN4 patients.

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## References

1 Brandi ML, Gagel RF, Angeli A, Bilezikian JP, Beck-Peccoz P, Bordi C, Conte-Devolx B, Falchetti A, Gheri RG, Libroia A, Lips CJ, Lombardi G, Mannelli M, Pacini F, Ponder BA, Raue F, Skogseid B, Tamburrano G, Thakker RV, Thompson NW, Tomassetti P, Tonelli F, Wells SA Jr, Marx SJ: Guidelines for diagnosis and therapy of men type 1 and type 2. J Clin Endocrinol Metab 2001;86:5658–5671.

2 Marx SJ: Molecular genetics of multiple endocrine neoplasia types 1 and 2. Nat Rev Cancer 2005;5:367–375.

3 Pellegata NS, Quintanilla-Martinez L, Siggelkow H, Samson E, Bink K, Hofler H, Fend F, Graw J, Atkinson MJ: Germ-line mutations in p27kip1 cause a multiple endocrine neoplasia syndrome in rats and humans. Proc Natl Acad Sci USA 2006;103:15558–15563.

4 Costa-Guda J, Marinoni I, Molatore S, Pellegata NS, Arnold A: Somatic mutation and germline sequence abnormalities in cdkn1b, encoding p27kip1, in sporadic parathyroid adenomas. J Clin Endocrinol Metab 2011;96:E701–E706.

5 Thakker RV: Multiple endocrine neoplasia type 1 (MEN1). Best Pract Res Clin Endocrinol Metab 2010;24:355–370.

6 Marx SJ: Hyperparathyroid genes: sequences reveal answers and questions. Endocr Pract 2011;3:18–27.

7 Melmed S: Mechanisms for pituitary tumorigenesis: the plastic pituitary. J Clin Invest 2003;112:1603–1618.

8 Rothenbuhler A, Stratakis CA: Clinical and molecular genetics of Carney complex. Best Pract Res Clin Endocrinol Metab 2010;24:389–399.

9 Daly AF, Tichomirowa MA, Beckers A: The epidemiology and genetics of pituitary adenomas. Best Pract Res Clin Endocrinol Metab 2009;23:543–554.

10 Verges B, Boureille F, Goudet P, Murat A, Beckers A, Sassolas G, Cougard P, Chambe B, Montvernay C, Calender A: Pituitary disease in MEN type 1 (MEN1): data from the France-Belgium MEN1 multicenter study. J Clin Endocrinol Metab 2002;87:457–465.

11 Platta CS, Mackay C, Welsh JS: Pituitary adenoma: a radiotherapeutic perspective. Am J Clin Oncol 2010;33:408–419.

12 Hofland LJ, Feelders RA, de Herder WW, Lamberts SW: Pituitary tumours: the sst/d2 receptors as molecular targets. Mol Cell Endocrinol 2010;326:89–98.

13 Debelenko LV, Brambilla E, Agarwal SK, Swalwell JI, Kester MB, Lubensky IA, Zhuang Z, Guru SC, Manickam P, Olufemi SE, Chandrasekharappa SC, Crabtree JS, Kim YS, Heppner C, Burns AL, Spiegel AM, Marx SJ, Liotta LA, Collins FS, Travis WD, Emmert-Buck MR: Identification of men1 gene mutations in sporadic carcinoid tumors of the lung. Hum Mol Genet 1997;6:2285–2290.

14 Fritz A, Walch A, Piotrowska K, Rosemann M, Schaffer E, Weber K, Timper A, Wildner G, Graw J, Hofler H, Atkinson MJ: Recessive transmission of a multiple endocrine neoplasia syndrome in the rat. Cancer Res. 2002;62:3048–3051.

15 Fero ML, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, Polyak K, Tsai LH, Broudy V, Perlmutter RM, Kaushansky K, Roberts JM: A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(kip1)-deficient mice. Cell 1996;85:733–744.

16 Morosetti R, Kawamata N, Gombart AF, Miller CW, Hatta Y, Hirama T, Said JW, Tomonaga M, Koeffler HP: Alterations of the p27kip1 gene in non-hodgkin's lymphomas and adult T-cell leukemia/lymphoma. Blood 1995;86:1924–1930.



Stratakis CA (ed): Endocrine Tumor Syndromes and Their Genetics.
Front Horm Res. Basel, Karger, 2013, vol 41, pp 63–78 (DOI: 10.1159/000345670)

17 Spirin KS, Simpson JF, Takeuchi S, Kawamata N, Miller CW, Koeffler HP: P27/kip1 mutation found in breast cancer. Cancer Res 1996;56:2400–2404.
18 Pappa V, Papageorgiou S, Papageorgiou E, Panani A, Boutou E, Tsirigotis P, Dervenoulas J, Economopoulos T, Raptis S: A novel p27 gene mutation in a case of unclassified myeloproliferative disorder. Leukemia Res. 2005;29:229–231.
19 Pasquali D, Circelli L, Faggiano A, Pancione M, Renzullo A, Elisei R, Romei C, Accardo G, Coppola VR, De Palma M, Ferolla P, Grimaldi F, Colao A, Colantuoni V: Cdkn1b v109g polymorphism a new prognostic factor in sporadic medullary thyroid carcinoma. Eur J Endocrinol 2011;164:397–404.
20 Landa I, Montero-Conde C, Malanga D, De Gisi S, Pita G, Leandro-Garcia LJ, Inglada-Perez L, Leton R, De Marco C, Rodriguez-Antona C, Viglietto G, Robledo M: Allelic variant at –79 (c>t) in cdkn1b (p27kip1) confers an increased risk of thyroid cancer and alters mRNA levels. Endocr Relat Cancer 2010;17:317–328.
21 Malanga D, De Gisi S, Riccardi M, Scrima M, De Marco C, Robledo M, Viglietto G: Functional characterization of a rare germline mutation in the gene encoding the cyclin-dependent kinase inhibitor p27kip1 (cdkn1b) in a Spanish patient with multiple endocrine neoplasia-like phenotype. Eur J Endocrinol 2012;166:551–560.
22 Agarwal SK, Mateo CM, Marx SJ: Rare germline mutations in cyclin-dependent kinase inhibitor genes in multiple endocrine neoplasia type 1 and related states. J Clin Endocrinol Metab 2009;94:1826–1834.
23 Georgitsi M, Raitila A, Karhu A, et al: Germline cdkn1b/p27kip1 mutation in multiple endocrine neoplasia. J Clin Endocrinol Metab 2007;92:3321–3325.
24 Belar O, De La Hoz C, Perez-Nanclares G, Castano L, Gaztambide S: Novel mutations in MEN1, CDKN1B and AIP genes in patients with multiple endocrine neoplasia type 1 syndrome in Spain. Clin Endocrinol 2012;76:719–724.
25 Molatore S, Marinoni I, Lee M, Pulz E, Ambrosio MR, degli Uberti EC, Zatelli MC, Pellegata NS: A novel germline CDKN1B mutation causing multiple endocrine tumors: clinical, genetic and functional characterization. Hum Mut 2010;31:E1825–E1835.
26 Tichomirowa MA, Lee M, Barlier A, et al: Cyclin-dependent kinase inhibitor 1b (CDKN1B) gene variants in AIP mutation-negative familial isolated pituitary adenoma kindreds. Endocr Relat Cancer 2012;19:233–241.
27 Marinoni I, Pellegata NS: p27kip1: a new multiple endocrine neoplasia gene? Neuroendocrinology 2011;93:19–28.
28 Sherr CJ, Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999;13:1501–1512.
29 Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF, Rolfe M: Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 1995;269:682–685.
30 Kamura T, Hara T, Matsumoto M, Ishida N, Okumura F, Hatakeyama S, Yoshida M, Nakayama K, Nakayama KI: Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(kip1) at G1 phase. Nat Cell Biol 2004;6:1229–1235.
31 Carrano AC, Eytan E, Hershko A, Pagano M: Skp2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat Cell Biol 1999;1:193–199.
32 Besson A, Hwang HC, Cicero S, Donovan SL, Gurian-West M, Johnson D, Clurman BE, Dyer MA, Roberts JM: Discovery of an oncogenic activity in p27kip1 that causes stem cell expansion and a multiple tumor phenotype. Genes Dev 2007;21:1731–1746.
33 Chu IM, Hengst L, Slingerland JM: The CDK inhibitor p27 in human cancer: Prognostic potential and relevance to anticancer therapy. Nat Rev Cancer 2008;8:253–267.
34 Molatore S, Kiermaier E, Jung CB, Pulz E, Höfler H, Atkinson MJ, Pellegata NS: Characterization of a naturally-occurring p27 mutation predisposing to multiple endocrine tumors. Mol Cancer 2010;9:116.
35 Payne SR, Kemp CJ: P27(kip1) (CDKN1B)-deficient mice are susceptible to chemical carcinogenesis and may be a useful model for carcinogen screening. Toxicol Pathol 2003;31:355–363.

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