p27kip1: A New Multiple Endocrine Neoplasia Gene?

Ilaria Marinoni  Natalia S. Pellegata

Institute of Pathology, Helmholtz Zentrum München, Neuherberg, Germany

Abstract

Multiple endocrine neoplasias (MEN) are autosomal dominant disorders characterized by the occurrence of tumors in at least two endocrine glands. Two types of MEN syndromes have long been known: MEN type 1 (MEN1) and MEN type 2 (MEN2), associated with a different spectrum of affected organs. MEN1 and MEN2 are caused by germline mutations in the MEN1 tumor suppressor gene and the RET proto-oncogene, respectively. Lately, a new type of MEN was identified (named MEN4) which is due to mutations in the CDKN1B gene, encoding for p27kip1 (p27), a cyclin-dependent kinase (Cdk) inhibitor that regulates the transition of cells from G1 to S phase. p27 is a non-canonical tumor suppressor since it is usually not somatically mutated in human cancers but it is often downregulated by post-translational mechanisms. The discovery of MEN4 has defined a new role for CDKN1B as a tumor susceptibility gene for multiple endocrine tumors. To date, six germline CDKN1B mutations have been found in patients with a MEN1-like phenotype but negative for MEN1 mutations. Due to the limited number of patients so far identified, the phenotypic features of MEN4 are not clearly defined. Here, we review the clinical and molecular characteristics of the MEN4 syndrome and summarize the main functions of p27 to better comprehend how their alteration can predispose to neuroendocrine tumors.

Key Words
Multiple endocrine neoplasia · p27kip1 · Cell cycle regulation

Introduction

Multiple endocrine neoplasias (MEN) are autosomal dominant syndromes characterized by tumors involving two or more neuroendocrine tissues. Two such syndromes have been clinically and genetically well characterized: the MEN type 1 (MEN1) and type 2 (MEN2). MEN1 is caused by loss-of-function mutations in the MEN1 tumor suppressor gene [1], and affected patients typically develop multiple parathyroid adenomas, pancreatic islet cell neoplasia, and anterior pituitary adenomas [2]. MEN2 is caused by activating germline mutations in the RET proto-oncogene [3] and, depending on the tumor spectrum, two subtypes are recognized: MEN2A, characterized by medullary thyroid carcinoma (MTC) and pheochromocytoma in about 20–50% of the cases, and by MTC and parathyroid adenoma in 5–20% of the cases [2]; MEN2B (formerly MEN3), presenting with MTC, pheochromocytoma (in 50% of the cases), marphanoid habitus and mucosal and digestive ganglioneuromatosis [4]. These syndromes have been reviewed in detail in other publications and will not be discussed in detail here [5–9].

Capitalizing on the discovery that a multiple endocrine neoplasia syndrome in the rat (named MENX) is caused by a germline loss-of-function mutation in Cdkn1b (encoding the cell cycle inhibitor p27), mutations in the human homologue CDKN1B were identified in few patients with multiple endocrine tumors and no germline mutations in the canonical susceptibility genes MEN1 and RET [10–13]. These findings lead to the identification
of a novel MEN syndrome, named MEN4 (OMIM No. 610755). The affected patients so far identified developed parathyroid and pituitary tumors, as well as other malignancies, but the spectrum of affected organs associated to MEN4 is still not clearly defined.

In this minireview, we provide a short overview of the role of p27 in normal and pathological states, a prerequisite to understand how alterations in p27 may associate with neuroendocrine tumor predisposition; we present clinical findings about the novel MEN4 syndrome and the functional characterization of the associated CDKN1B mutations.

**Short Summary of the Functions of p27 and Their Regulation**

p27 is a member of the KIP/CIP family of cyclin/cyclin-dependent kinase (Cdk) inhibitors and regulates cell cycle progression at the G1 to S phase transition of the cell cycle. p27 was first identified as inhibitor of the cyclin E/Cdk2 complex in cells arrested in G1 by TGF-β treatment [14], and was later found to inhibit both cyclin E/Cdk2 and cyclin A/Cdk2 complexes. The main target of Cdk2 is the pRb protein: upon phosphorylation, pRb releases members of the E2F family of transcription factors which in turn allow for the transcription of genes required for the progression to the S phase. Thus, binding of p27 to cyclin E/Cdk2 prevents pRb phosphorylation and stops the cells in G1 (fig. 1).

In normal cells, the activity of p27 is tightly regulated at different levels: transcriptional, translational and post-translational. Transcriptional regulation of the Cdkn1b promoter [15, 16] and control of mRNA translation [17] have both been reported, but the best known mechanism regulating p27 function is post-translational and regulates its abundance: proteolysis via the ubiquitin-proteasome pathway [18]. In quiescent cells, p27 accumulates in the nucleus where it binds to cyclin/Cdk complexes to block cell cycle progression [19]. Upon mitogenic stimulation, the cells need to re-enter the cell cycle and p27 undergoes rapid proteasome-mediated degradation following two main pathways. The first involves the ubiquitylation-promoting complex KPC1 and takes place in the cytoplasm [20]. At the beginning of the G1 phase, after the dissociation from the cyclin E/Cdk2 complex, p27 is phosphorylated at Serin 10 (Ser10), the major phosphorylation site of p27. Phosphorylation at Ser10 increases the binding affinity of p27 for CRM1 (exportin 1). This association of p27 to CRM1 promotes the nuclear export of

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**Fig. 1.** Graphic representation of the nuclear interactions of p27. Upon mitogenic stimulation, p27 is released from the cyclin E/Cdk2 complex. The dissociation from p27 activates Cdk2, which in turn phosphorylates the Rb protein. Phosphorylated Rb releases the transcription factor E2F which induces the expression of genes required for the G1 to S phase progression.
In the cytoplasm, p27 is ubiquitylated by the KPC ubiquitin ligase and degraded by the proteasome. The second degradation pathway requires p27 phosphorylation at the threonine (Thr) 187 residue by cyclin E/Cdk2 complexes and takes place in the nucleus in late G1. The phosphorylation at Thr187 creates a recognition site for the SKP2 ubiquitin ligase which induces p27 poly-ubiquitylation and subsequent degradation by the proteasome [22] (fig. 2).

Subcellular localization plays a role in regulating p27 function, not only by rendering the protein available to the various degradation pathways, but also because there is evidence that p27 exerts different functions while in the nucleus or in the cytoplasm. While in the nucleus p27 inhibits cyclin A, E/Cdk2 complexes, in the cytoplasm p27 is a necessary assembly factor for cyclin D/Cdk4,6 complexes, which in turn promote cell proliferation. Moreover, cytoplasmic p27 is involved in cell cycle-independent functions such as apoptosis and cell motility. The contribution of p27 to both these processes is cell type-dependent and the molecular mechanisms involved are not completely understood.

Overexpression of p27 induces apoptosis in several cancer cell lines [23], including HeLa cells where p27 stimulates caspase-3 activation [24]. In contrast, p27 prevents apoptosis of mouse mesangial cells upon growth factor removal, since cells from p27−/− animals show higher level of apoptosis than those from wild-type littermates [25]. Similarly, a dual behavior is also displayed by p27 in the regulation of cell motility, since it either promotes or prevents cell migration in a cell type-dependent manner. Wild-type p27 can directly associate with and inhibit the activity of RhoA, a GTPase protein known to be involved in stress fiber formation. Inhibition of RhoA stimulates cell motility [26]. In keeping with this finding, mouse fibroblasts lacking p27 (p27−/−) show in-
increased RhoA activity and migrate poorly [26] (fig. 3). It has also been shown that ectopic overexpression of p27 stimulates the migration of hepatocellular carcinoma cells by similar mechanisms [27, 28]. In contrast, p27 negatively regulates migration of vascular smooth muscle cells, umbilical vein endothelial cells, and oral cancer cells [29–32]. These differences possibly originate from cell type-specific variation in the relative balance between RhoA (negative regulator of cell motility) and Rac (positive regulator of cell motility) activity.

It is important to mention that p27 is also involved in cell differentiation. Indeed, p27 controls the development of the mouse cerebral cortex by regulating in a concerted manner cell cycle exit, differentiation of cortical progenitor cells into neurons and migration of neurons to the cortical plate [33]. In particular, p27 promotes the differentiation of cortical progenitors into neurons by up-regulating the expression of neurogenin 2, one of the principal basic helix-loop-helix factors that drives neuronal differentiation [33]. Furthermore, it has been demonstrated that p27 cooperates with p57Kip2 (belonging to the same family of Cdk inhibitors) in regulating the cell cycle exit and differentiation in the lens, placenta and pituitary gland [34, 35]. These functions are independent from the ability of p27 to inhibit cyclin/Cdk complexes.

In conclusion, p27 is a complex molecule whose multiple functions are regulated through a plethora of protein-protein interactions and post-translational modifications, alteration of intracellular localization and efficiency of degradation.

**p27 and the Classical MEN Genes**

The focus of this review being the link between p27 and neuroendocrine tumor predisposition, it is relevant to explore the interactions between p27 and the canonical genes responsible for MEN-associated tumor susceptibility, i.e. MEN1 and RET. Recent studies discovered that the protein encoded by the Men1 gene, named Menin, directly regulates the expression of p27 [36]. Specifically, Menin associates with members of the trithorax group of transcription factors, such as the mixed-lineage leukemia (MLL), MLL2 and Ash2 to form a nuclear complex that is able to methylate histone H3 at lysine 4 thereby activating transcription [37]. Menin recruits this multi-protein complex with histone methyltransferase activity to the Cdkn1b promoter, promoting its transcription in mouse pancreatic islet cells [38]. As a consequence of the Menin-

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**Fig. 3.** Graphic representation of the cytoplasm interactions of p27. In the cytoplasm, p27 interacts with RhoA and inhibits the activity of the RhoA/Rock in actin polymerization, thereby promoting migration. p27 also binds Grb2, a small GTPase involved in Ras activation. The modality of Ras activation by p27 is poorly understood.
mediated transcriptional activation of Cdkn1b, pancreatic islet tumors that develop in mice heterozygous for a Men1-null allele, where the wild-type Men1 allele is somatically lost, show no expression of p27 [38].

The link between p27 and RET has not yet been thoroughly explored. It was shown that inhibition of oncogenic RET activity results in down-regulation of p27 in thyroid medullary carcinoma cells [39], likely through the phosphorylation of the transcription factor AFX/FKHR by the serin-threonine kinase AKT [40]. The in vitro induction of a constitutively active MEN2A-specific RET mutant isofrom (RET2A) correlates with reduced p27, and elevated cyclin D protein levels, leading to an increase in Cdk activity, pRb phosphorylation and proliferation, even under growth arrest conditions. RET2A expression also correlates with decreased CDKN1B mRNA levels [41]. Consistent with the in vitro results, immunohistochemistry of MEN2A-associated human pheochromocytoma shows decreased levels of p27 and increased levels of cyclin D1 as compared to normal adrenal tissue [41].

The above findings indicate that p27 is an important readout of the Menin signaling pathway and a downstream target of oncogenic RET in endocrine cells. It is tempting to speculate that p27 may represent a critical nodal point where various signals converge to ultimately regulate neuroendocrine cell proliferation.

**Roles of p27 in Tumorigenesis**

A drastic reduction of p27 expression has been observed in almost 50% of all human cancers and it usually correlates with their histological aggressiveness and with poor patient outcome [42, 43]. While this is consistent with a tumor suppressor role for p27 [42, 44], CDKN1B does not behave like a canonical tumor suppressor gene because it is rarely mutated (only 3 somatic variants so far reported [45–47]) or lost (usually hemizygous loss of 12p13, where CDKN1B maps) [48] in human tumors. Studies comparing in situ hybridization of CDKN1B mRNA and immunohistochemical staining for p27 performed on the same tumor biopsies revealed that samples with high or low p27 immunoreactivity have similar CDKN1B mRNA levels [49, 50]. These observations support the hypothesis that downregulation of p27 in human tumors is mainly due to post-transcriptional or post-translational mechanisms rather than genetic alterations. In keeping with this hypothesis, it has been shown that enhanced proteasome-mediated degradation is associated with decreased p27 expression in colon cancer [51]. Thus, at the somatic level, a reduction of p27 associates with tumorigenesis.

A number of animal models have demonstrated that lack of functional p27 promotes tumor formation. p27-null mice develop intermediate lobe pituitary adenomas (frequency 100%) [52] and p27 deficiency cooperates with other oncogenic events to promote tumor formation in different mouse organs [53, 54]. Similarly, MENX-affected rats, displaying extremely reduced/absent p27 expression in their tissues due to the causative Cdkn1b mutation, develop multiple endocrine tumors [10].

Noteworthy, a 50% reduction in p27 protein amount predisposes heterozygous p27+/− mice to tumors in multiple organs when combined either with DNA damage or with additional oncogenic events such as activation of the Trk-t1 oncogene [56] or loss of the tumor suppressor Pten [54]. Molecular analysis of tumors developing in these p27+/− mice showed that the remaining wild-type allele is neither mutated nor silenced [55]. Thus, p27 is a dose-dependent tumor suppressor in mice [55]. The fact that reduction of p27 expression, rather than complete loss, is usually detected in human tumors has led to the speculation that p27 is a dose-dependent tumor suppressor also in human tumors [55]. However, neuroendocrine tumors might be an exception (see below).

As mentioned above, downregulation of p27 occurs frequently in human tumors. One of the mechanisms used by the tumor cells to impair the p27-mediated regulation of cell cycle progression is the cytoplasmic sequestration of the protein. Indeed, cytoplasmic localization of p27 has been correlated with high tumor grade, poor prognosis and higher metastatic potential in carcinomas of the breast, cervix, esophagus and uterus, as well as in some lymphomas and leukemias [57]. As mentioned earlier, in addition to not being able to interact with cyclin/Cdk complexes, cytoplasmic p27 possesses several ‘pro-oncogenic’ functions. In this cellular compartment p27 plays a role in cytoskeleton remodeling, apoptosis and migration, thereby potentially influencing tumor invasiveness and metastasis formation [57]. The existence of cyclin/Cdk-independent pro-oncogenic functions of p27 was further proven by studies of a mouse strain expressing a mutant p27 unable to bind to cyclin/Cdk complexes (p27CK−). Homozygous p27 CK−/CK− mice are prone to a higher tumor multiplicity than homozygous p27−/− mice and this widespread tumorigenesis was also observed in p27+/CK− heterozygous mice indicating that this mutant p27 behaves as a dominant oncogene in vivo [58]. The authors also observed a correlation between the cytoplas-
mic localization of p27 and the abnormalities arising in the corresponding tissues, suggesting that the oncogenic role played by p27 likely depends on the cytoplasmic localization of the protein [58].

Mechanistically, retention of p27 in the cytoplasm of human tumor cells is mediated by AKT-dependent phosphorylation of the protein at Thr157. Therefore, the activated AKT pathway (an event which frequently occurs in various tumors) translates into sequestration of p27 in the cytoplasm and loss of its cell cycle inhibitory activity, as observed in breast cancer [59–61].

A distinct mechanism by which the tumor cells reduce p27 activity is the overexpression of the SKP2 ubiquitin ligase, which then promotes p27 degradation, as reported in breast and prostate cancer [62, 63].

**Role of p27 in Neuroendocrine Tumors**

p27-null mice develop intermediate lobe pituitary adenomas (incidence 100%) as the sole tumor phenotype, suggesting that pituitary cells are particularly sensitive to defects in cell cycle regulation. Due to the phenotype of these mice, human pituitary tumors have been extensively investigated for CDKN1B genetic/epigenetic alterations and p27 expression. Similarly to that reported for several nonendocrine tumors, no somatic mutations in CDKN1B were found. Methylation of the Cdkn1b promoter was observed in rat GH3 and mouse GHRH-CL1 pituitary tumor cell lines [64] but not in primary pituitary human adenomas [65]. A significant reduction of nuclear p27 protein expression was observed in all subtypes of pituitary tumors compared to normal pituitary tissue, while it is completely absent in corticotrope adenomas and malignant pituitary tumors [66, 67]. As it occurs in other tumor types, the level of CDKN1B mRNA was similar between normal tissue and pituitary adenoma, suggesting that the reduced protein level in tumor cells might be due to accelerated degradation that removes p27 from the nucleus. This hypothesis is corroborated by the increase in p27 phosphorylation at Thr187 observed in pituitary corticotrope adenomas, which then promotes p27 degradation by the SKP2-SCF complex [66]. Increased phosphorylation at Thr187 was often due to high SKP2 protein expression, establishing an inverse correlation between these two proteins which has also been observed in nonendocrine tumors. Moreover, overexpression and activation of the AKT kinase in pituitary tumors compared to normal pituitary tumors were identified [68]. AKT phosphorylates p27 on Thr157 and promotes retention of the protein in the cytoplasm. Altogether, these data suggest that p27 plays a role in the development of human sporadic pituitary adenoma.

Other types of neuroendocrine tumors have also been analyzed for p27 expression. Reduced p27 levels have been found in over 50% sporadic pheochromocytoma [69] and in poorly differentiated gastro-enteropancreatic neuroendocrine carcinomas and metastatic carcinomas, where it correlated with high proliferation and poor prognosis [70, 71]. Parathyroid adenomas (primary hyperparathyroidism) and secondary hyperplastic glands also exhibit aberrantly reduced expression of p27 [72].

**CDKN1B Mutations and the Novel MEN4 Syndrome**

The identification of a homozygous frameshift mutation in p27 as the causative genetic mutation associated with the rat MENX syndrome demonstrated for the first time that Cdkn1b is a tumor susceptibility gene for multiple endocrine tumors. Following this discovery, genetic changes in the human homologue CDKN1B have been sought in patients showing a MEN-like phenotype, but negative for mutations in MEN1 and RET, and to date six germline mutations have been identified [10–13] (table 1).

The first CDKN1B mutation was identified in a 48-year-old Caucasian female affected by a GH-secreting pituitary tumor (acromegaly) and hyperparathyroidism [10]. The mutation is a germline heterozygous TGG-TAG nonsense mutation at codon 76 which determines the premature truncation of the protein at this residue (p27W76X). Interestingly, the same variant (W76X) has been found as a somatic change in the tumor cells of an adult leukemia/lymphoma patient [45]. A sister of the mutation-positive proband carries the same mutation and has been diagnosed with renal angiomyolipoma (a MEN1-associated tumor). Following transfection in Rat2 (rat fibroblasts) and MCF7 (human breast cancer) cell lines, the mutant p27W76X protein localizes mainly to the cytoplasm [10]. Although the molecular analysis of the renal angiomyolipoma of the mutation-positive sister revealed no loss of heterozygosity at the CDKN1B locus, the tumor tissue showed no p27 staining. Quantitative RT-PCR showed that the CDKN1B mRNA level is similar in tumor and normal tissue of this patient [Pellegata, unpubl. data], suggesting that the absence of protein is due to post-transcriptional modification. The functional characterization of this mutant may help understand why the only tumor tissue available for immunohistochemistry from a p27W76X mutation-positive individual showed no protein expression, indicating a lack of both wild-type and mutated p27 proteins.
A second germline CDKN1B mutation was identified in a Dutch patient diagnosed with three lesions compatible with a diagnosis of MEN1: small-cell neuroendocrine cervical carcinoma, ACTH-secreting pituitary adenoma (Cushing’s disease), and hyperparathyroidism [11]. This mutation is a 19-bp duplication which leads to a premature stop at codon 69 generating a truncated protein. The cervical carcinoma of the patient showed loss of the wild-type allele of CDKN1B and lack of p27 protein expression. Biallelic inactivation of CDKN1B is an exceedingly rare condition in human tumors, which usually exhibit hemizygous loss of p27, as mentioned earlier. So, the finding that tumors in CDKN1B mutation carriers show loss of heterozygosity or lack of p27 expression suggests that p27 may behave as a ‘canonical’ tumor suppressor in neuroendocrine cells. Analysis of additional tumors of mutation-positive patients will help address this issue.

Three new potentially pathogenic mutations were recently discovered in patients showing a MEN1-like phenotype or familial primary hyperparathyroidism (1°HPT) but without MEN1 mutations [12]. A mutation at the −7 position in the Kozak sequence (ATG−7G>C) was found 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. In vitro studies showed that the ATG−7G>C variant associates with reduction in p27 protein amount (as demonstrated by transient transfections in HEK293 cells), suggesting that a haploinsufficient expression of the protein might be the mechanism implicated in tumor predisposition in this patient.

A second individual affected by primary hyperparathyroidism (1°HPT) and displaying masses in both duodenum and pancreas was found to carry a missense mutation at codon 95, CCC>TCC, leading to a Pro>Ser (P95S) protein change. This change occurs in a proline-rich region (amino acid 91–95) of p27 which constitutes the binding site of the protein to Grb2, a protein which in complex with SOS recruits the proto-oncogene Ras and converts it from the inactive GDP-bound form to the active GTP-bound form. In GST pull-down assays, the p27P95S missense variant showed reduced binding to Grb2. Although the effect of the association of p27 with Grb2 on downstream pathways is not totally understood, impaired binding between the two proteins might influence the Ras/mitogen activated protein kinase (MAPK) signal transduction pathway activation (fig. 3) [72].

The third variant changes the stop codon in glutamine (TAA>CAA; stop>Q), and leads to a protein predicted to be 60 amino acids longer than the wild-type one. This mutation was found in a patient with a family history of 1°HPT [12]. The stop>Q variant associates with reduc-
tion in the encoded p27 protein amount in vitro upon transfection in HEK293 cells. So, as for the ATG–7G>C variant, a low amount of p27 may predispose to neuroendocrine tumors.

Recently, we have identified a variant at codon 69 (c.678C>T, p.P69L) in a patient presenting with multiple typical bronchial carcinoids, 1°HPT, papillary thyroid carcinoma with neck lymph node metastasis, microadenoma in the pituitary gland and bilateral multiple lung metastasis [13]. In vitro experiments showed that the p27P69L mutant protein is more unstable than the wild-type one. Considering the crystal structure of the Cdk2-CyclinA-p27 complex, the P69L change affects one of the six amino acids involved in the direct binding of p27 to CDK2 [74]. Cdk2 pull down assay performed in HeLa cells transfected with the P69L variant showed that this protein does not bind to the kinase [13].

Altogether, these findings brought about the recognition of a new rare type of MEN syndrome caused by mutation of CDKNIB and named MEN4. The CDKNIB changes so far identified either affect the localization, the stability or the protein binding abilities of p27 (table 1).

The phenotypic features associated with MEN4 are still undefined due to the small number of patients reported.

Conclusions

The recognition of both the MENX (rat) and the MEN4 (human) syndromes has identified Cdkn1b/CDKNIB as a new tumor susceptibility gene for multiple neuroendocrine tumors. These findings, together with previous analysis of animal models with defective p27 function, point to a critical role for p27-mediated cell cycle regulation in neuroendocrine cells homeostasis. We speculate that in these cells aberrant/absent p27 activity cannot be compensated by other cyclin/Cdk inhibitors as it occurs in tissues unaffected by p27 malfunction. This would explain the tissue-selective tumorigenesis associated to p27 germline mutations. The identification and functional characterization of additional CDKNIB mutations will broaden our understanding of the relationship between p27 and neuroendocrine tumor predisposition. In addition, knowing the properties of the mutant p27 proteins associated to hereditary tumors may facilitate the development of targeted therapeutic strategies for a more effective clinical management of the patients carrying those mutations and of their families.

Acknowledgments

We thank the members of the laboratory for stimulating discussions. This work was in part supported by the Deutsche Krebshilfe (Grant 109223 to N.S.P).

References

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