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ORIGINAL ARTICLE

Human pheochromocytomas show reduced p27Kip1 expression that is not associated with somatic gene mutations and rarely with deletions

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Abstract Pheochromocytomas are neuroendocrine tumors arising in the neural crest-derived chromaffin cells of the adrenal gland or in extra-adrenal sympathetic ganglia (paragangliomas). In a rat model of multiple endocrine neoplasia (MEN), absence of functional p27Kip1 protein predisposes to pheochromocytoma and paraganglioma development. As no data is available regarding the involvement of p27Kip1 in human pheochromocytoma and/or paraganglioma, we set out to determine the expression pattern of p27Kip1 in those tumor types. A panel of 25 pheochromocytomas and 23 paragangliomas was collected. Two pheochromocytomas were from MEN2 patients. The paragangliomas included 15 tumors that developed at the carotid bifurcation, three in the jugulo–tympanic area, and five at other sites. Except for the MEN2 cases, all others were apparently sporadic. Immunohistochemistry for p27Kip1 and the proliferation marker Ki67 was performed. We found that p27Kip1 expression is reduced/lost in 56% of pheochromocytomas, but only in 18.1% of paragangliomas. Downregulation of p27Kip1 was not associated with increased proliferation. Cases showing reduced/lost p27Kip1 expression were screened for the presence of somatic mutations in *CDKN1B* (p27Kip1) and for allelic imbalance at the p27Kip1 locus. Three cases had allelic imbalance but none had mutations. In conclusion, pheochromocytomas display extreme reduction/loss of p27Kip1 expression at high frequency.

Keywords Pheochromocytoma · Paraganglioma · p27Kip1 · Ki67 · Immunohistochemistry · Allelic imbalance

Abbreviations

CDK	cyclin-dependent kinase
MEN	multiple endocrine neoplasia
VHL	von Hippel–Lindau
SDH	succinate dehydrogenase
AI	allelic imbalance

Introduction

Pheochromocytomas are neuroendocrine tumors that arise from neural crest-derived chromaffin cells of the adrenal gland and extra-adrenal sympathetic ganglia (referred to as paragangliomas). About 25% of patients with pheochromocytoma and/or paraganglioma present with a family history of von Hippel–Lindau disease (VHL), multiple endocrine neoplasia type 2 (MEN2), one of the three familial paraganglioma syndromes (PGL1, PGL3, PGL4), or, more rarely, neurofibromatosis type 1 (NF1). The causative germline mutations in these syndromic cancers have been identified in *VHL*, rearranged during transfection (*RET*), succinate dehydrogenase (*SDH*) subunits B, C, D, and *NF1*, respectively [14]. Approximately 10–15% of pheochromocytomas/paragangliomas are malignant and present very challenging problems for therapy. Currently, the malignant

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status of pheochromocytomas/paragangliomas is established primarily by the presence of metastases at non-chromaffin sites [18], and although several markers have been proposed to predict malignancy in those tumors, there is no consensus as to the existence of histological, clinical, or molecular indicators of worse prognosis.

We have identified a MEN-like syndrome in the rat (named MENX) that shows phenotypic overlap with both MEN1 and MEN2 human syndromes [21]. Affected rats develop bilateral pheochromocytoma and parathyroid adenoma, multifocal anterior pituitary adenoma, and thyroid C-cell hyperplasia, as well as paraganglioma [21]. The histopathology of the rat lesions resembles that of their human counterpart [21]. Noteworthy, pheochromocytoma has complete penetrance in MENX rats: Adrenal medullary hyperplasia is already evident at 2 months of age and progresses to pheochromocytoma with age. We recently mapped the *MenX* locus [35] and identified the genetic mutation that causes MENX. Affected rats harbor a germline loss-of-function mutation of *Cdkn1b*, a gene in which germline mutations predisposing to cancer had not been previously reported [34]. Noteworthy, we also identified a mutation-negative, suspected MEN1 patient who carries a germline heterozygous nonsense mutation in the human *CDKN1B* gene [34]. *Cdkn1b* encodes the cyclin-dependent kinase (Cdk) inhibitor p27Kip1, an important cell-cycle regulatory protein that controls the progression from G1 to the S phase by interacting with cyclinE/Cdk2 and cyclinD1/Cdk4 complexes [36, 39]. Emerging evidence suggests that p27Kip1 can regulate cellular functions other than cell-cycle progression, such as cell differentiation and migration [6, 8]. Due to its central role in many important cellular processes, p27Kip1 has been analyzed in several human malignancies. The *CDKN1B* gene, encoding p27Kip1, usually is not somatically mutated in human cancers and is inactivated through epigenetic mechanisms only in a small subset of tumors including lymphoid malignancies and hepatocellular carcinoma [27, 31]. In contrast, p27Kip1 expression level is frequently reduced or absent in human tumors, and the downregulation of p27Kip1 is a statistically significant predictor of survival and tumor behavior in a variety of cancers, including prostate, breast, hepatocellular, and gastrointestinal cancers (reviewed in [28]). In the tissues of MENX-affected rats, the level of p27Kip1 protein is extremely reduced or absent compared to wild-type animals [34], and likely, this inactivation triggers tumor development in neuroendocrine tissues. Due to the tumor spectrum of MENX rats with inactivation of p27Kip1, we decided to study the expression pattern of p27Kip1 in human pheochromocytoma and paraganglioma. In this paper, we report that p27Kip1 expression is frequently reduced/lost in pheochromocytoma (56%) but less so in paraganglioma (18.1%; P=0.0151, phaeochromocytoma versus paraganglioma). This suggests that, despite their histopathological similarities, those two tumor types follow distinct molecular pathways. The downregulation of p27Kip1 represents the single most-frequent molecular alteration so far reported in pheochromocytoma, implicating p27Kip1 in the pathogenesis of this tumor type.

Materials and methods

Samples

Formalin-fixed, paraffin-embedded tissues from 25 pheochromocytomas and 22 paragangliomas were retrieved from the archives of the Institute of Pathology, Technical University of Munich. The histological classification of the cases was reviewed and is reported in Tables 1 and 2. Of the pheochromocytoma cases, two belonged to MEN2A patients (PC21 and PC22) and were both bilateral and associated with medullary thyroid carcinoma (MTC); one case (PC20) was associated with a renal cell carcinoma (RCC; Table 1), but the patient was negative for *VHL* gene mutations. The paragangliomas included 13 tumors that developed at the carotid bifurcation, three in the jugulo–tympanic area, and 6 in other sites (Table 2). All cases, except PC21 and PC22, were apparently sporadic.

Immunohistochemistry

Conventional sections of the tissue samples mentioned above were used for immunohistochemistry. Immunohistochemistry was performed using monoclonal antibodies against p27Kip1 (clone 57, 1:1,000, BD Transduction Laboratories, San Jose, CA, USA) and the proliferation marker antigen Ki-67 (MIB-1, 1:500, DAKO, Carpinteria, CA, USA). Sections were cut at 3 μM and stained immunohistochemically using an automated immunostainer (Ventana Medical System, Tucson, AZ, USA) according to previously published procedures [37]. Positive controls (tonsils) were included in each staining run to confirm the adequacy of the staining. The degree of staining was evaluated using a semi-quantitative scoring system at ×200 magnification. To score the p27Kip1 staining, both the intensity of the staining and the percentage of positive tumor cells were taken into consideration. Three positive signs indicate >80% of tumor cells showing strong nuclear staining for p27Kip1, two positive signs indicate >80% of tumor cells showing very weak nuclear positivity, one positive sign indicate very weak to moderate positivity in <20% of tumor cells, and the negative sign indicate that tumor cells have negative staining. Only distinct nuclear staining of tumor cells was used for scoring the Ki67 immunoreactivity, which was determined semiquantitatively and is indicated as the percent of positive cells on the entire

Table 1 Clinicopathological, immunohistological, and molecular characteristics of pheochromocytomas

Case ID	Age/sex	p27Kip1 staining	Ki-67 (%)	p27Kip1 sequence	AI
PC1	64/M	++	<5		
PC2	49/F	+	<5		
PC3[a]	32/M	+	<5	Ex1+387 c/t	–
PC4[a]	50/M	+++	<5		
PC5	68/F	+[b]	<5		D12S391
PC6	40/F	+++[b]	<5		
PC7	58/M	+	<5	Ex1+387 c/c; G109G	D12S1580
PC8	70/F	+	<5	Ex1+387 c/c	
PC9	74/M	–	<5	Ex1+387 c/t	–
PC10	48/M	+++	<5		
PC11	41/F	–	<5		–
PC12	54/F	+	<5		–
PC13	54/M	+++	<5		
PC14	68/F	+++	<5		
PC15	70/M	+++	<5		
PC16[a]	49/F	–	<5	Ex1+387 c/c	–
PC17	76/M	+++	<5		
PC18	44/M	+++	<5		
PC19	73/F	+++	<5		
PC20[c]	64/F	+	<5		–
PC21[d]	45/F	+++	<5		
PC22[d]	31/F	+[b]	<5		–
PC23	66/F	+++	<5		
PC24	73/F	+[b]	<5	Ex1+387 c/c; V109G	
PC25	64/F	++	<5		–

Cases in bold were sequenced for the 5′ region and coding exons of the *CDKN1B* (p27Kip1) gene. p27Kip1 polymorphisms were named according to the SNP500Cancer database: Ex1+387 c/t corresponds to SNP rs34330 and V109G to SNP rs2066827. Except when indicated, cases were apparently sporadic.

AI Allelic imbalance, *M* male, *F* female, *negative sign* no AI

[a] Vascular invasion

[b] Some cytoplasmic staining

[c] Tumor associated to RCC

[d] MEN2A

tumor represented in the section. The immunohistochemistry of the clinical data in all cases was reviewed blindly by two pathologists (LQ-M and FF). Images were recorded using a Hitachi camera HW/C20 installed in a Zeiss Axioplan microscope with Intellicam software (Zeiss MicroImaging, Thornwood, NY, USA). Adobe PhotoShop (San Jose, CA, USA) was used for image processing.

Molecular analyses

Normal and tumor tissue areas were grossly macrodissected from five 5-μm sections of formalin-fixed, paraffin-embedded tissue blocks to obtain homogeneous cell populations. The tumor tissue samples contained >80% tumor cells. DNA was extracted from both cell populations as follows. Briefly, tissues were deparaffinized using xylene and ethanol, centrifuged, and dried. Pellets were resuspended in 200 μl of lysis buffer (0.05 M/l Tris–HCl at pH 7.5, 1 mM/l ethylenediaminetetraacetic acid (EDTA), 0.5% Tween-20) with 1 mg/ml proteinase K. After overnight digestion at 55°C, samples were boiled for 10 min to inactivate the proteinase K and used for polymerase chain reaction (PCR) amplification. Sequencing of the complete coding region and 140 bp of the 5′ untranslated region of the *CDKN1B* gene was performed with the Big-Dye terminator kit (Applied Biosystems), and sequences were run on an ABI377 sequencer. Primers for sequencing the 5′ region and exon 1 were: p27Kip1_1a FW GTCGGGGTCT GTGTCTTTTG, p27Kip1_1a Rev CCATGTCTCTG CAGTGCTTC; p27Kip1_1b FW TGTCTAACGG GAGCCCTAGC, p27Kip1_1b Rev AGTAGAACTCGGG CAAGCTG; p27Kip1_1c Fw AGTTAACCCGGGACTTG GAG, p27Kip1_1c Rev GTCCGACGGATCAGTCTTTG; p27Kip1_1d Fw AGGAGAGCCAGGATGTCAGC, p27Kip1_1d Rev GCCAGGTAGCACTGAACACC. Primers for exon 2 were: p27Kip1_2 Fw CTGACTATGGGGCCAA CTTC, p27Kip1_2 REV GCCAGCAACCAGTAAGATCAG. Sequencing of the *VHL* gene was performed using previously

Table 2 Clinicopathological, immunohistological, and molecular characteristics of paragangliomas

Case ID	Location	Age/sex	p27Kip1 staining	Ki-67 (%)	p27Kip1 sequence	AI
PG1[a]	Nasal sinus	67/F	+	>20	Ex1+387 c/t	D12S391,D12S358D12S1580
PG2	Cbt	70/F	+++	<5		
PG3	Rtp	63/F	++[b]	<5		–
PG4_1[a]	Cbt	40/F	+++	<5		
PG4_2	Recurrence of PG4_1	45	+++	<5		
PG4_3	Recurrence of PG4_1	48	+++	<5		
PG5	Cbt	61/F	+++	<5		
PG6	Cbt	40/F	+++	<5		
PG7[a]	Cbt	38/F	+++	<5		
PG8	Cbt	69/F	+++	<5		
PG9	Cbt	59/M	+++	<5		
PG10	Jtt	59/F	+++	<5		
PG11	Cbt	48/F	–	<5	Ex1+387 c/t	–
PG12	Base of skull	62/M	+++	<5		
PG13	Cbt	62/M	+++	<5		
PG14	Jtt	62/M	+++	<5		
PG15	Mediastinal	54/F	+++	<5		
PG16	Cbt	34/M	+++	<5		
PG17	Cbt	40/M	+++[b]	<5		
PG18	Retrosternal	54/M	+++	<5		
PG19	Rtp	61/M	+++	<5		
PG20	Cbt	71/F	+++	<5		
PG21	Jtt	52/F	–	<5	Ex1+387 c/c	
PG22	Cbt	38/F	+++	<5		

Cases in bold were sequenced for the 5′ region and coding exons of the *CDKN1B* (p27Kip1) gene. p27Kip1 polymorphisms were named according to the SNP500Cancer database: Ex1+387 c/t corresponds to SNP rs34330 and V109G to SNP rs2066827. All cases were apparently sporadic.
AI Allelic imbalance, *Cbt* carotid body tumor, *Rtp* retroperitoneal tumor, *Jtt* jugulotympanic tumor, *M* male, *F* female, *negative sign* no AI
[a] Local infiltration
[b] Some cytoplasmic staining

reported primers and conditions [40]. Allelic imbalance (AI) analysis at the *CDKN1B* locus was performed using the chromosome 12 p markers D12S391, D12S358, and D12S1580 in cases showing abnormal p27Kip1 staining. PCR products were loaded onto a 377XL sequencer (PE Biosystems). Allele size and fluorescent intensity were determined by Genescan and Genotyper software (PE Biosystems). AI was established by determining the fluorescent intensity of each allele and calculating their ratio in both normal (*N*) and tumor (*T*) DNA using the formula (allele1−*N*/allele2−*N*)/(allele1−*T*/allele2−*T*). Samples were scored as showing AI if an allelic ratio of <0.67 or >1.5 was obtained [10].

Statistical analyses

The number of patients with a reduced expression level of p27Kip1 and that of patients showing the typical high expression was compared between the two tumor types (pheochromocytoma versus paraganglioma cases) using the Fisher's exact test. Two sided P value<0.05 was considered statistically significant.

Results

Expression patterns of p27Kip1 and Ki-67 in pheochromocytoma

Normal adrenal medulla typically shows strong nuclear positivity for p27Kip1 in virtually all cells and low percentage of Ki67-positive cells (below 5%), indicating low proliferative activity (data not shown). From the 25 analyzed pheochromocytomas, 11 cases (44%) showed strong nuclear p27Kip1 staining in >80% of the tumor cells (classified as '+++,' Fig. 1a, b; Table 1), while 14 cases (56%) showed a very weak (Fig. 1c–f) to negative nuclear staining for p27Kip1 (Fig. 1h). The case illustrated in Fig. 1g, showed complete loss of p27Kip1 staining except for one small nodular area of the tumor, which had very strong nuclear expression of the protein. In the negative cases, infiltrating lymphocytes and residual normal adrenal gland were strongly stained with p27Kip1 and, thus, served as internal positive controls for the adequacy of staining. The immunohistochemical staining was mainly nuclear also in the cases with reduced p27Kip1 protein

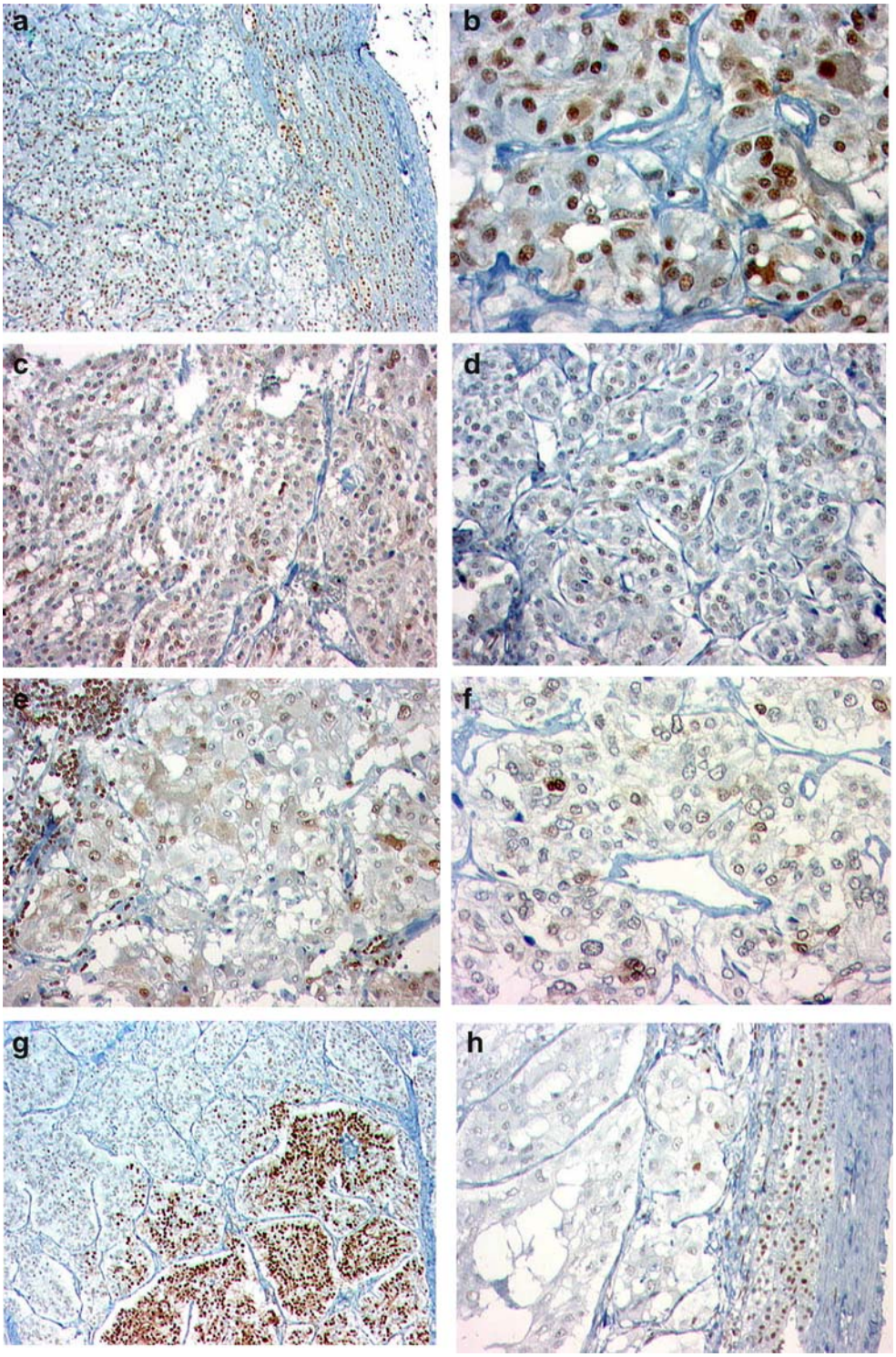

Fig. 1 Immunohistochemical analysis of pheochromocytoma. Sections were scored for the p27Kip1 staining intensity, and the percentage of stained cells in the tumor (see "Materials and methods"). **a**, **b** Strong p27Kip1 staining (+++). **a** Note on the right side that the residual normal adrenal cells are strongly positive for p27Kip1 (100×). **b** Higher magnification demonstrates that the vast majority of the tumor cells have a strong, nuclear positivity for p27Kip1 (400×). **c**–**f** Cases with reduced expression of p27Kip1. **c**, **d** Weak diffused expression of p27Kip1 in the majority of the cells (++; 200×). **e**, **f** Very weak or moderate expression of p27Kip1 in <20% of the cells (+; **e** 200×, **f** 320×). Note that in case **e**, in addition to the reduced expression of p27Kip1, there is also cytoplasmic staining. On the upper-left side of **e**, there are numerous lymphocytes with strong nuclear positivity used as internal control. **g**–**h** Cases of pheochromocytoma with lost p27Kip1 expression or negative for p27Kip1 (−). **g** Only one nodule within the tumor showed nuclear positivity for p27Kip1; the rest of the tumor was negative (100×). **h** Pheochromocytoma negative for p27Kip1. On the right side, there are residual normal adrenal cells strongly positive for p27Kip1 used as internal control (200×)

expression. Four cases (PC5, PC6, PC22, PC24) showed in addition some cytoplasmic reactivity (as an example, see Fig. 1e). The extent and distribution of the cytoplasmic p27Kip1 staining mirrored the nuclear staining in each sample; i.e., when the nuclear staining was very faint and present in only a few cells, the same also held true for the cytoplasmic staining. All pheochromocytomas showed immunoreactivity for Ki-67 in <5% of the cells (Table 1) irrespective of p27Kip1 status.

Expression pattern of p27Kip1 and Ki-67 in paraganglioma

Of the 22 paraganglioma cases, 18 (77.3%) showed strong nuclear immunoreactivity in >80% of the tumor cells

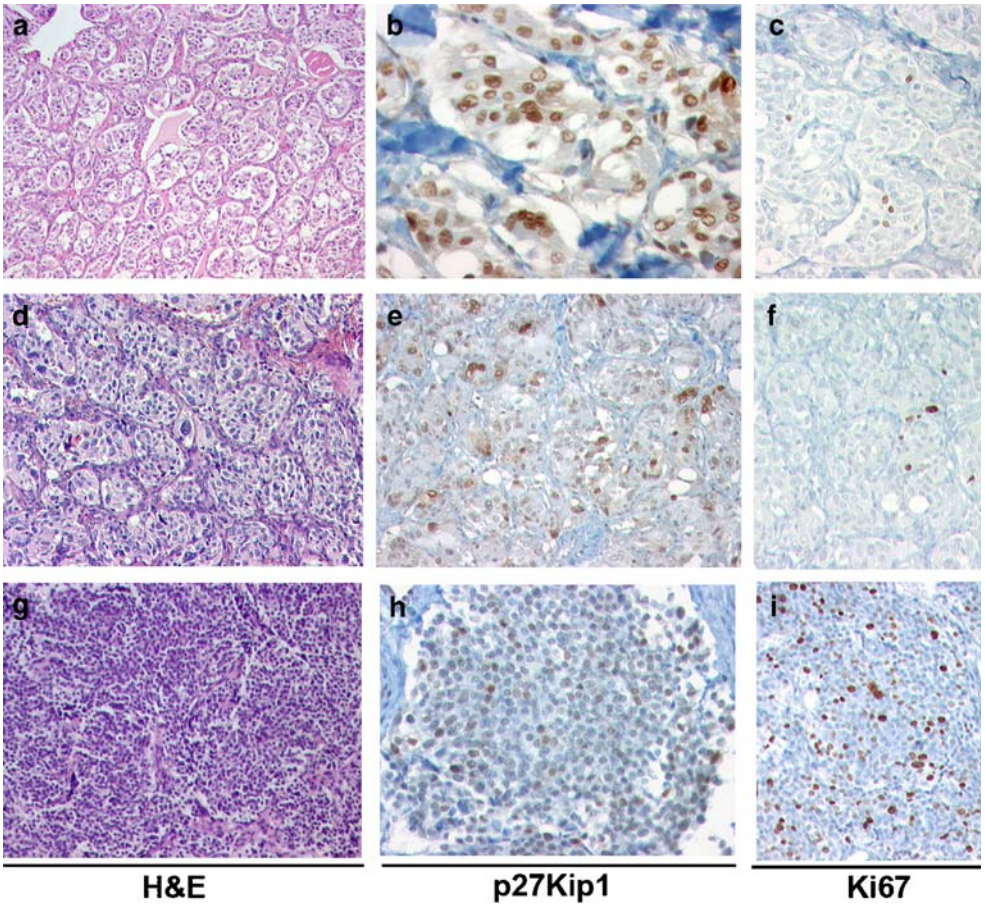

Fig. 2 Histological and immunohistochemical analysis of paraganglioma. **a**–**c** H&E stain of a paraganglioma (**a** 100×) with strong p27Kip1 nuclear positivity (+++) in virtually all tumor cells (**b** 400×) and very low proliferation rate as demonstrated by the Ki67 immunoreactivity (**c** 200×). **d**–**f** H&E stain of a paraganglioma (**d** 160×) with weak to moderate p27Kip1 staining in >80% (++) of the tumor cells (**e** 100×) and very low proliferation rate (**f** 200×). **g**–**i** H&E stain of the aggressive paraganglioma case PG1 (**g** 100×) with p27Kip1 staining in less than 20% (+) of the tumor cells (**h** 400×) and high proliferation rate (**i** 200×)

(Fig. 2b), while 4 cases (18.1%) had weak (Fig. 2e and h) or negative p27Kip1 staining (Table 2). The staining was mainly nuclear also in the cases with reduced p27Kip1 positivity. Only two cases (PG3 and PG17) showed some cytoplasmic reactivity for p27Kip1, and the extent and distribution of the cytoplasmic p27Kip1 staining mirrored the nuclear staining in each sample (as described above for the PC cases). Paraganglioma cases with abnormal p27Kip1 staining included tumors at different locations throughout the body (Table 2). All paraganglioma cases showed immunoreactivity for Ki-67 in <5% of the cells, except one (PG1) that showed positivity in more than 20% of the cells (Fig. 2i). PG1 was classified as an aggressive paraganglioma, as it was a tumor in the nasal cavity associated with infiltration into the base of the skull, both frontal sinuses, and both orbitae.

Genetic analyses of the samples showing abnormal p27Kip1 immunoreactivity

The reduction in the level of expression of p27Kip1 in these tumors (both phaeochromocytoma and paraganglioma cases) could be due to loss of the chromosomal region harboring the gene. Therefore, we searched for allelic imbalance (AI) at the p27Kip1 locus using three highly polymorphic microsatellite markers (D12S391, D12S358, D12S1580) flanking the p27Kip1 locus on chromosome 12p13 (Fig. 3). These markers present a centromere-to-telomere orientation, covering 890 Kb of chromosome 12 that include the *CDKN1B* gene (encoding p27Kip1). The 5′ end of the *CDKN1B* gene is approximately 230 kb downstream of D12S358 and the 3′ end 472 kb upstream of D12S1580. For ten (out of 14) pheochromocytomas and 3 paragangliomas with reduced/lost p27Kip1 expression (see Tables 1 and 2), normal tissue was available for AI analyses. Two samples were non-informative for D12S391, four were non-informative for D12S358, and two for D12S1580. The remaining samples were informative for at least one marker in the region (Fig. 3a). One pheochromocytoma showed AI for D12S391 (PC5; Fig. 3b), another showed AI for D12S1580 (PC7), and the aggressive paraganglioma (PG1) showed AI for all three markers (not shown). Overall, the AI frequency calculated as the ratio of samples showing AI versus the total number of informative cases for each marker is quite low, ranging from 11% (D12S358) to 18% (D12S391, D12S1580).

Nine cases showing a decreased expression of p27Kip1 were analyzed for the presence of somatic mutations in the coding region of the *CDKN1B* gene (Tables 1 and 2). Control DNA from the adjacent normal tissues was also

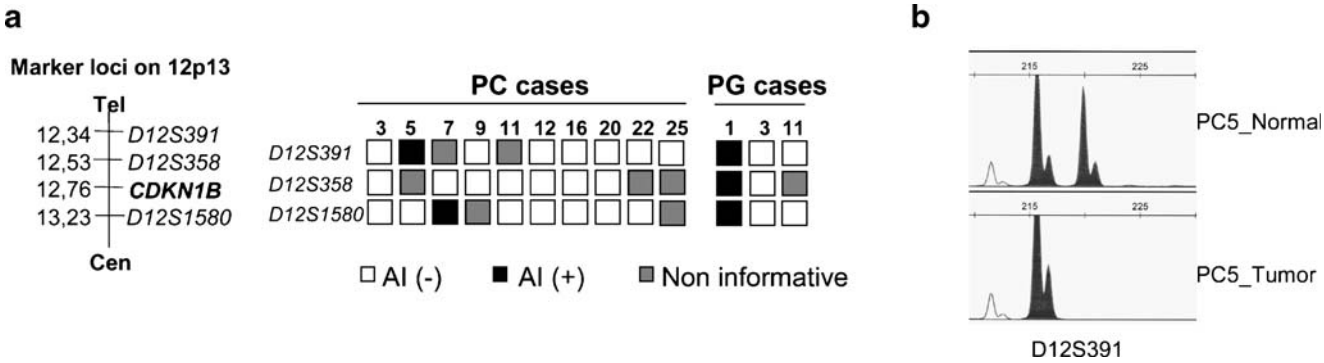

Fig. 3 Allelic imbalance analysis for markers at 12p13. **a** The panel summarizes the status of thirteen cases having abnormal p27Kip1 immunohistochemical staining. Cases described as retaining heterozygosity; having AI and non-informative cases are indicated by *open*, *close*, and *gray* squares, respectively. On the left is reported the position of the markers along chromosome 12 in megabases [according to the National Center for Biotechnology Information (NCBI) genome browser], as well as the position of the *CDKN1B* gene encoding p27Kip1. PG1 was the only sample showing AI for all the markers tested. PC5 and PC7 showed AI for one marker each. **b** Example of AI for marker D12S391 in sample PC5. The normal DNA shows two alleles, while the tumor DNA retains the smaller allele but has lost the larger one

sequenced. PCR amplification of the 5′ region and the two coding exons was performed followed by direct automated sequencing of the PCR products. Although potential pathogenic mutations in *CDKN1B* were not found, known polymorphisms in the 5′ untranslated region of exon1 (SNP rs34330) and at codon 109 (Val109Gly, SNP rs2066827) were identified (Tables 1 and 2).

Patient PC20 could be affected by VHL disease, as the tumor spectrum of this syndrome includes RCC and pheochromocytoma. Therefore, we sequenced the *VHL* gene in the DNA from the normal tissue of case PC20 but we found no mutations. Furthermore, no mutations were identified in *MEN1* and *RET* in this patient (data not shown).

Statistical analysis

The number of patients with a reduced expression level of p27Kip1 (scored −, +, or ++) or showing the high p27Kip1 expression typical of normal tissues (scored +++) was compared between the two tumor types (pheochromocytoma versus paraganglioma) using the Fisher's exact test. The results showed that the number of pheochromocytoma samples having reduced p27Kip1 expression is significantly higher than that of paraganglioma cases showing reduced p27Kip1 positivity (P=0.0151). Age at presentation, malignant potential, and tumor location (for the paragangliomas) were not found to be significantly associated with downregulation of p27Kip1 in both tumor types.

Discussion

In this study, we examined the expression of the cyclin-dependent kinase inhibitor p27Kip1 in a panel of apparently sporadic pheochromocytoma and paraganglioma cases. Two syndromic pheochromocytomas (MEN2) were among the cases analyzed. A striking decrease/loss of p27Kip1 expression was observed at a high frequency in pheochromocytoma (56% of cases), as demonstrated by the low immunostaining and decreased number of cells/field stained with the anti-p27Kip1 antibody. In the remaining cases, the typical strong nuclear expression was observed. In contrast to the high frequency of decreased p27Kip1 expression observed in pheochromocytoma, only 18.1% of the paragangliomas showed reduction of p27Kip1 immunoreactivity. These two tumor types share many similarities: They both arise from chromaffin cells derived from the embryonal neural crest, they are histologically very similar, and they both can be caused by germline mutations in the same susceptibility genes (*VHL*, *SDHD*, *SDHB*). However, our data points to a different genetic etiology of those tumor types with a significantly higher percentage of downregulation of p27Kip1 in pheochromocytomas compared to the paragangliomas (P=0.0151). This finding strongly implicates reduction of p27Kip1 in adrenal but less so in extra-adrenal pheochromocytoma development. These observations are in agreement with our studies on the MENX rat model system. Indeed, affected rats (homozygous for a loss-of-function mutation in p27Kip1 [34]) develop pheochromocytoma with complete penetrance at young age (<6 months), while paragangliomas occur later in life (>10 months), and their incidence is only about 10% (Pellegata, unpublished observation).

In contrast to what is observed by immunohistochemistry for many antigens, we did not find a continuum of p27Kip1 immunoreactivity in our samples, but instead a discontinuous expression pattern. Indeed, we observed samples with virtually all tumor cells highly positive for p27Kip1 (as seen in normal adrenal chromaffin cells), while the others had a dramatic reduction of positivity in either the number of stained tumor cells and/or the intensity of staining.

The molecular mechanism underlying the reduction/loss of p27Kip1 expression is as yet uncertain. We have examined allelic status of chromosome 12p, where the *CDKN1B* gene is located, but changes occur at a low incidence in these tumors: One aggressive paraganglioma showed loss of p27Kip1 through allelic imbalance (AI), and two pheochromocytomas showed AI at markers in the vicinity of p27Kip1. The overall incidence of AI at the analyzed loci (D12S391, D12S358, D12S1580) was 11–18% (calculated as the ratio of AI/informative cases for each marker). Although microdeletions are a formal possibility, it is likely that AI is not the most important mechanism for loss of protein expression. In addition, we did not find mutations in the coding sequence of the *CDKN1B* gene. Both these results are in agreement with the reported low incidence of AI at the p27Kip1 locus and the scarcity of somatic mutations in *CDKN1B* in other solid tumors [24, 25, 30, 38]. It has been shown that the intracellular level of the p27Kip1 protein is mainly regulated at the post-translational level via proteasome-mediated degradation [33]. Indeed, in most cases of decreased p27Kip1 concentrations, increased proteolytic activity toward the protein has been observed [12, 19, 29]. In contrast, no changes in the level of *CDKN1B* messenger RNA (mRNA) have been found in tumors that show downregulation of the p27Kip1 protein [11, 23, 29]. Our studies on the p27Kip1-negative tissues of MENX rats [34] have also failed to detect changes in the level of p27Kip1 mRNA. Thus, the mechanism causing loss of p27Kip1 expression in human pheochromocytomas and paragangliomas might be post-translational.

Cytoplasmic relocation due to phosphorylation or sequestration by partner proteins plays an important role in regulating p27Kip1 functions. It has been proposed that, while p27Kip1 in the nucleus acts primarily as a tumor suppressor by modulating cyclin-cdk complexes, cytoplasmic p27Kip1 has oncogenic properties. Indeed, expression of a p27Kip1 protein that can only localize in the cytoplasm was recently found to increase motility, survival, and Akt levels in MCF7 breast cancer cells [42]. Cytoplasmic p27Kip1 staining has been observed in several tumor types and is in general associated with more aggressive behavior and poorer prognosis [3]. Among the samples analyzed, six showed cytoplasmic staining for p27Kip1 in addition to nuclear staining (see tables). Four of these cases (PC5, PC6, PC24, PG3) had an overall downregulation of p27Kip1 expression (three classified as "+" and one as "++"), indicating that, in those samples, cytoplasmic relocation could contribute to the loss of p27Kip1 tumor suppressor function.

PC21 and PC22 are MEN2A-associated tumors arising in patients who carry a germline mutation in the *RET* protooncogene. It has been reported that younger age of onset, multifocality of the tumors, and the presence of extra-adrenal tumors is parameters usually associated with a genetic predisposition to pheochromocytoma [32]. Thus, cases PC3 (32 years of age) and PC11 (41 years) could also be syndromic pheochromocytomas. Interestingly, three of the above cases (PC3, PC11, PC22) showed reduction/loss of p27Kip1. This observation warrants further studies in a larger cohort of syndromic pheochromocytomas to determine whether p27Kip1 inactivation may occur at a higher incidence in hereditary cases.

The biological consequences of the downregulation of p27Kip1 in pheochromocytoma are not yet known. Except one, all the tumors we analyzed have relatively low proliferative activity (Ki67 immunoreactivity, <5%) irrespective of the p27Kip1 expression level. The fact that downregulation of p27 does not cause massive increase in cell proliferation is in agreement with the phenotype of MENX-affected rats. These animals display increase in overall body size and weight compared to their normal littermates and show a remarkable increase in the size of some organs [34], but the proliferative activity of most tissues (including neuroendocrine tissues) is similar to that observed in unaffected animals (Pellegata, unpublished). It is, therefore, likely that impaired p27Kip1 function may affect not only proliferation but additional cellular processes required to maintain tissue homeostasis (such as differentiation and senescence).

Our observation that reduction of p27Kip1 expression in pheochromocytoma does not increase tumor cell proliferation is in contrast to what has been reported for other tumor types (including medulloblastomas, endocrine tumors of the pancreas, and gastrointestinal tract and most lymphoma subtypes) in which immunoreactivities for p27Kip1 and Ki67 are inversely correlated [1, 9, 13]. When an inverse relationship exists, it is not easy to discern whether the low p27Kip1 expression is the pathological cause or the physiological consequence of increased proliferative activity. In our cases, the decreased p27Kip1 concentration is not the result of an increased proliferation of the tumor cells, but it is due to an altered regulation of the protein's expression. Studies performed in mice have shown that neuroendocrine cells are especially sensitive to defects in cell-cycle regulatory proteins affecting the Rb pathway. In fact, mice heterozygous for Rb [22, 26], chimeric for Rb −/− [41], and deficient for the Cdk-inhibitors p18Ink4c and p27Kip1 develop tumors with high penetrance in the pituitary gland, pancreatic islets, and thyroid [20]. Hyperplasia of the adrenal medulla also develops in mice chimeric for Rb −/− [41]. Our results on the frequent reduction of p27Kip1 in human adrenal medullary tumors further support the hypothesis that this protein plays a role in regulating endocrine tissue homeostasis, not necessarily via controlling cell proliferation.

While the molecular mechanisms underlying the development of hereditary pheochromocytoma and paraganglioma

have been largely elucidated, advances in understanding the molecular pathogenesis of their sporadic counterparts has lagged behind. Somatic mutations in the six known pheochromocytoma/paraganglioma susceptibility genes occur at relatively low incidence (10–25%) in sporadic tumors [5, 7]. Candidate gene approaches have identified tumor suppressor genes inactivated in pheochromocytomas, such as the RAS-association domain family 1, isoform A (*RASSF1A*), and the p16 tumor suppressor genes. *RASSF1A* [2, 4] and p16 [17] were found to be down regulated through promoter region hypermethylation in 22–48% and in 24% of pheochromocytomas, respectively. Interestingly, the incidence of p16 and of *RASSF1A* inactivation was higher in hereditary pheochromocytomas compared to sporadic cases [2, 4, 17]. Recently, genome-wide approaches have been used to identify molecular pathways involved in sporadic and hereditary pheochromocytomas and paragangliomas development [15, 16, 18]. These studies have not yet provided us with individual candidate genes to further analyze. The limited knowledge of the molecular pathogenesis of sporadic pheochromocytomas and paragangliomas has hampered the identification of predictors of malignancy and the development of novel therapeutic approaches to target specific dysregulated pathways. In view of the newly established role of p27Kip1 downregulation in pheochromocytomas, cell cycle regulatory pathways can now be further explored in this tumor type, as well as new therapeutic modalities able to restore p27Kip1 expression (function) in adrenal medullary tumor cells.

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