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Adrenergic Differentiation and Ret Expression in Rat Pheochromocytomas

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Abstract Pheochromocytomas are catecholamine-producing tumors of the adult adrenal medulla. They are rare in humans and most other species but common in laboratory rats. However, the relevance of rat pheochromocytomas as a model for their human counterparts is uncertain. Previous studies of spontaneous and drug-induced rat pheochromocytomas and the PC12 pheochromocytoma cell line suggested a distinctive noradrenergic phenotype, possibly reflecting origin from a progenitor not present in the adult human adrenal. In this study, we studied 31 pheochromocytomas derived from test and control male and female rats in toxicologic studies for expression of the epinephrine-synthesizing enzyme phenylethanolamine-*N*-methyltransferase (PNMT) and the receptor tyrosine kinase Ret. PNMT, which defines adrenergic chromaffin cells, is frequently expressed in human pheochromocytomas, often in tumors that also overexpress RET. We also tested for the expression of the cell cycle checkpoint protein $p27^{Kip1}$, which recently was reported absent in pheochromocytomas from a strain of rats with a hereditary mixed multiple endocrine neoplasia (MEN)-like syndrome. Using immunoblots, we demonstrated PNMT expression in almost 50% of the 31 tumors, although often at lower levels than in normal rat adrenal medulla. The majority of tumors overexpressed Ret. There was no apparent correlation between PNMT and Ret. However, in this study, PNMT expression was strongly associated with tumors arising in female rats, while overexpression of Ret did not show a sex predilection. Robust expression of $p27^{Kip1}$ was seen in all tumors from the toxicologic studies and also in a small sample of pheochromocytomas from Long–Evans rats, which were reported to have a mixed MEN-like syndrome in the 1980s. The present results show that rat pheochromocytomas have greater phenotypic diversity than previously believed and greater similarity to their human counterparts with respect to these two important markers. Loss of $p27^{Kip1}$ does not appear to account for the high frequency of pheochromocytomas in commonly utilized rat strains.

Keywords p27kip 1 · phenylethanolamine *N*-methyltransferase · pheochromocytoma · Ret proto-oncogene

Pheochromocytomas are catecholamine-producing tumors of the adrenal medulla. They are rare tumors in humans and most other species. The majority of human pheochromocytomas occur sporadically, while up to 30% are manifestations of hereditary tumor syndromes [1]. Subsets of both sporadic and syndrome-associated human pheochromocytomas exhibit distinctive phenotype markers [2, 3]. However, the mechanisms underlying the expression of different phenotypes are unknown [4].

An important distinction between subsets of human pheochromocytomas is the expression of an adrenergic or noradrenergic phenotype, defined, respectively, by the presence or absence of phenylethanolamine *N*-methyltransferase (PNMT), the enzyme that synthesizes epinephrine from norepinephrine [3]. Human pheochromocytomas also frequently overexpress the receptor tyrosine kinase, RET, either with a variety of *RET* gene mutations in multiple endocrine neoplasia (MEN) syndromes 2A or 2B [5] or as wild type in sporadic tumors [6]. Human pheochromocyto-

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mas that overexpress RET are frequently adrenergic, and one possible explanation for the association is the origin of adrenergic pheochromocytomas from a distinctive RET-positive progenitor.

In contrast to other species, rats show an exceptionally high frequency of pheochromocytomas. The tumors often develop spontaneously in aging rats. In addition, many toxicological studies have shown that the frequency of pheochromocytomas in rats is increased by a variety of genotoxic or nongenotoxic agents [7]. The relevance of this easy inducibility to human pathobiology is unclear because there are no known inducers of pheochromocytomas in humans. However, rats are a potentially rich source of information that may contribute to the understanding of cellular mechanisms that underlie distinctive phenotypes.

Previous studies of spontaneous or drug-induced rat pheochromocytomas suggested that the tumors are usually [8, 9], though not always [10], noradrenergic. There is little or no information on Ret expression by these tumors in most previous studies. However, the widely studied PC12 cell line of rat pheochromocytoma cells is noradrenergic [11] and expresses extremely low levels of Ret [12], which is of the wild type.

We recently performed microarray-based gene expression profiling of rat pheochromocytomas that arose in several studies of unrelated drugs and chemicals conducted by the National Toxicology Program. It is surprising to note that the expression of PNMT messenger ribonucleic acid (mRNA) was apparently preserved in a substantial number of the tumors, several of which also overexpressed Ret [13]. This might have simply suggested that previous studies were not sufficiently representative of different rat pheochromocytoma phenotypes. However, PNMT is distinctive among the catecholamine biosynthetic enzymes in often showing discordance between the expression of its mRNA and protein [14], and most of the previous studies were based on immunohistochemical staining for the PNMT protein. The present investigation was therefore undertaken with several objectives: to corroborate the expression of PNMT at the protein level, to correlate the expression of PNMT with the expression of Ret, and to determine whether either marker is associated with specific toxicologic insults or distinctive tumor histology.

A further objective was motivated by the recent discovery of a hereditary predisposition to pheochromocytomas as part of a mixed MEN syndrome in Sprague–Dawley rats with a germline mutation of the *Cdkn1b* gene, which encodes the cell cycle checkpoint protein $p27^{kip1}$ [15]. The mutation was found to prevent the expression of p27 in the rat tumor tissues through a posttranscriptional mechanism. A partially overlapping tumor syndrome had been described in the 1980s in Long–Evans hooded rats [16], but the genetic defect was never identified. The genetic factors predisposing rats to develop pheochromocytomas in toxicologic studies are also unknown. We therefore sought to determine whether loss of p27 characterizes the pheochromocytomas that arise both in toxicological studies and in the Long–Evans model.

Materials and Methods

Tumors and Normal Adrenal Glands

Rat pheochromocytoma tumor tissue from 31 tumors arising in separate animals was procured from the National Toxicology Program. The tumors, which were previously utilized in a gene expression-profiling study [13], represented both male and female rats from several toxicological studies, including control and test animals receiving a variety of pharmacologically unrelated compounds as shown in Table 1. All tumors arose in adult animals. All of the tumors were blotted and probed for Ret and PNMT. Representative tumors were blotted and probed for $p27^{kip1}$. Scanned images of hematoxylin-and-eosin-stained histologic sections of all of the tumors were examined for possible correlations of histological features with Ret and/or PNMT expression. In addition, unstained sections that were available from ten tumors were utilized to stain immunohistochemically for PNMT and $p27^{kip1}$. We were unable to stain for Ret because the staining of paraffin-embedded rat tissues for Ret, in our experience, is greatly affected by fixation conditions [17], which were not optimal.

In addition to the tumors from the toxicological studies, in a search of archival material at Tufts New England Medical Center, we were able to retrieve sections of five pheochromocytomas from Long–Evans hooded rats described in the 1982 study [16]. We also stained these immunohistochemically for PNMT and $p27^{kip1}$.

Histological sections of intact normal rat adrenals were immunohistochemically stained similarly to the tumor sections.

Tissue Isolation and Immunoblots

Protein extraction and immunoblotting were as previously described [18]. Pooled normal adrenal medullary tissue from adult F344 rats was extracted similarly to tumor tissue and resolved on the same blots. To minimize adrenal cortical contamination of the samples of normal medulla, bisected adrenals were placed under a dissecting microscope, and the gray medullary tissue was carefully dissected away from the surrounding yellow cortex using no. 11 scalpels, which have thin, pointed tips. Proteins were resolved on precast 4–15% polyacrylamide gradient gels (Bio-Rad, Hercules, CA, USA), to permit multiple proteins

Table 1 Treatments of rats from which pheochromocytoma tissue was derived

Treatment	Rat strain			
	F344		R2	
	Male	Female	Male	Female
Control	2	5	4	0
Butyl benzyl phthalate	1			
2,2-*Bis*(bromomethyl)-1,3-propanediol	1			
t-Butylhydroquinone			1	
Codeine		2		
Isobutyl nitrate	2			
Manganese sulfate			1	
Nickel subsulfide	1			
Salicylazosulfapyridine	2			
Scopolamine	1	2		
1-Trans-delta-9-tetrahydrocannabinol	3	1	1	
Vanadium pentoxide	1			
Total	14	10	7	0

The population represents banked tumor tissue from separate toxicological studies of the indicated agents. Control animals were those that spontaneously developed pheochromocytomas in the same studies. The sexes and strains of the animals and the numbers of tumors under each treatment condition reflect the availability of the banked tissue and the designs of the separate studies, not true tumor frequencies.

with a wide range of molecular weights to be retained in the gel and probed for on the same blot. Blots were probed either with a pan-Ret antibody using an affinity-purified rabbit antibody directed against c-Ret (R787, Immuno-Biological Laboratories, Tokyo, Japan, catalog no. 18121) or with separate antibodies against Ret 9 and Ret 51, two forms of Ret that are differentially expressed in human pheochromocytomas [19] and that utilize somewhat different signaling pathways [20] (antibodies provided by Dr. Brian Tsui-Pierchala). PNMT was detected using a rabbit antibody raised and validated in our laboratory as previously described [17]. A mouse monoclonal antibody (clone SX53G8, DakoCytomation) was used to detect $p27^{kip1}$. Actin and/or neuron-specific enolase were probed as loading controls (antibodies from Santa Cruz biologicals, Santa Cruz, CA, USA, and Polysciences, Warrington, PA, USA). Bands were visualized using a chemiluminescence reporter system as previously described [17], and relative band intensities were calculated using Kodak Image 1D software.

Immunohistochemistry

Immunohistochemical staining for PNMT or $p27^{kip1}$ was performed on paraffin sections using the same antibodies employed for immunoblots (anti PNMT, 0.5 μg of IgG/ml, anti p27, 110 μg of IgG/ml). Incubation with primary antibody was performed overnight at 4°C after microwave antigen retrieval in citrate buffer, pH 6.0, and was followed by secondary antibody conjugated to polymer-bound horseradish peroxidase (Biocare Medical, Concord, CA, USA). Immunoreactivity was visualized using diaminobenzidine as the chromogen. Normal rabbit or mouse IgG was substituted for the primary antibody as a negative control.

Results

Immunoblots

All of the tumors from the toxicology studies were blotted and probed for Ret and PNMT. Ten representative tumors were blotted and probed for $p27^{kip1}$. Expression of Ret and/or PNMT was detected in more than half of the tumors from both rat strains studied. Ret was often expressed at higher levels in the tumors than in normal adrenal medulla on the same blots. In contrast, PNMT, when detectable at all, was often present at lower than normal levels. This characteristic has also been noted in adrenergic pheochromocytomas of humans and other species [7]. All tumors studied expressed $p27^{kip1}$ (Fig. 1). There were no obvious differences between tumors from the R2 and F344 strains. Therefore, because the number of R2 tumors was limited, data from the two strains were pooled for further analysis. For the same reasons, data were pooled for all tumors arising in animals treated with drugs or toxins, for comparison to pooled controls.

To correlate PNMT and Ret expressions and to compare the expression between different groups of animals, tumors were arbitrarily defined as PNMT positive if PNMT band intensities on immunoblots were greater than or equal to 20% of intensities of bands from normal adrenal medulla on the same blots. They were classified as positive for overexpression of Ret if Ret band intensities on immunoblots were greater than or equal to three times the intensities of bands from normal adrenal medulla on the same blots. These cutoffs were chosen to be within the ranges employed in other proteomic and gene expression profile studies and to be amenable to clear discrimination on the blots, based on our previous experience. We also tested the

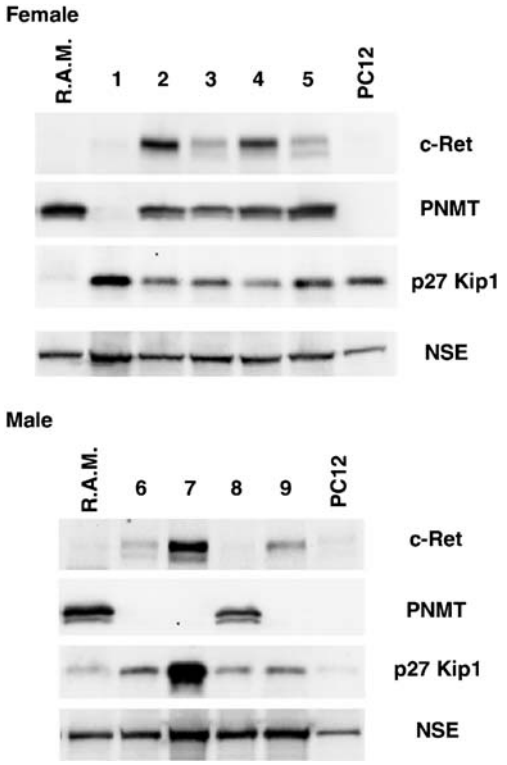

Fig. 1 Immunoblots comparing expression of Ret and PNMT in representative pheochromocytomas from female (*tumors 1–5*) or male (*tumors 6–9*) rats, normal rat adrenal medulla (*R.A.M.*) and the PC12 rat pheochromocytoma cell line. These blots were derived from the first nine tumors studied and, by chance, represent the apparent preferential expression of PNMT in tumors from females as shown in the entire series of 31 tumors in Table 3. "Neuron-specific" enolase (*NSE*) is expressed in normal and neoplastic chromaffin cells but not in adrenal cortical cells and therefore serves to confirm that all lanes contain comparable amounts of adrenal medullary or tumor tissue [17]

more stringent threshold of PNMT band intensity greater than 50% the intensity of bands from normal adrenal medulla. Comparisons were assessed by Chi-squared tests, with statistical significance of $P<0.05$. The results are tabulated in Tables 2 and 3.

Table 2 Numbers of tumors overexpressing Ret and/or maintaining PNMT expression in pooled untreated or treated rats (Ret greater than or equal to threefold higher than normal adrenal medulla; PNMT greater than or equal to 20% of expression in normal adrenal medulla)

	Spontaneous tumors		Tumors from treated animals	
	Male	Female	Male	Female
Ret−/PNMT−	1		1	
Ret−/PNMT+			1	
Ret+/PNMT−	4		8	1
Ret+/PNMT+	1	5	4	4
Totals	6	5	14	5

Spontaneous tumors were those arising in the control animals in Table 1.

Table 3 Numbers and percentages of tumors overexpressing Ret or maintaining PNMT expression in all male or female rats pooled from control and treated cohorts (Ret ≥3 fold higher than in normal adrenal medulla; PNMT ≥20% of expression in normal adrenal medulla)

	Male (%)	Female (%)
PNMT		
−	15 (71)	1 (10)
+	6 (29)	9 (90)
Total	21 (100)	10 (100)
Ret		
−	3 (14)	0 (0)
+	18 (86)	10 (100)
Total	21 (100)	10 (100)

$P=0.005$ for male versus female, Chi-squared test, or 0.002, Fisher's exact test
$P=0.53$ for male versus female, Chi-squared test or Fisher's exact test

Tabulation of the separate data for the control and treated groups revealed two major findings (Table 2). First, the majority of tumors overexpressed Ret in both groups. Second, there were no obligate relationships between Ret and PNMT, as all possible combinations of the two were seen. However, the numbers of rats were too small to permit meaningful comparisons of the frequencies of different combinations for the control and treated groups. In contrast, highly significant differences were detected when tumors from male and female rats were analyzed separately (Table 3). Although tumors from both males and females both frequently overexpressed Ret ($P>0.5$), there was a striking association of PNMT expression with tumors from females ($P<0.005$). This association was also observed using the more stringent threshold of PNMT band intensity greater than 50% the intensity of bands from normal adrenal medulla (70% of females versus 19% of males, $P<0.005$).

To determine whether the tumors that overexpressed Ret could be distinguished by differential expression of Ret 9 versus Ret 51, eight tumors representing each of the possible combinations of Ret and PNMT listed in Table 2 were blotted and probed for the two separate forms of Ret. Tumors that overexpressed Ret expressed either or both forms in varying proportions (not shown), and there were no apparent correlations with concomitant presence or absence of PNMT.

Immunohistochemistry

Sections of ten tumors from the toxicology studies that were PNMT positive ($n=5$) or negative ($n=5$) on immunoblots were stained immunohistochemically for PNMT to determine the correlation between the two methods. Complete correlation was observed in that tumors considered positive on immunoblots were also positive immuno-

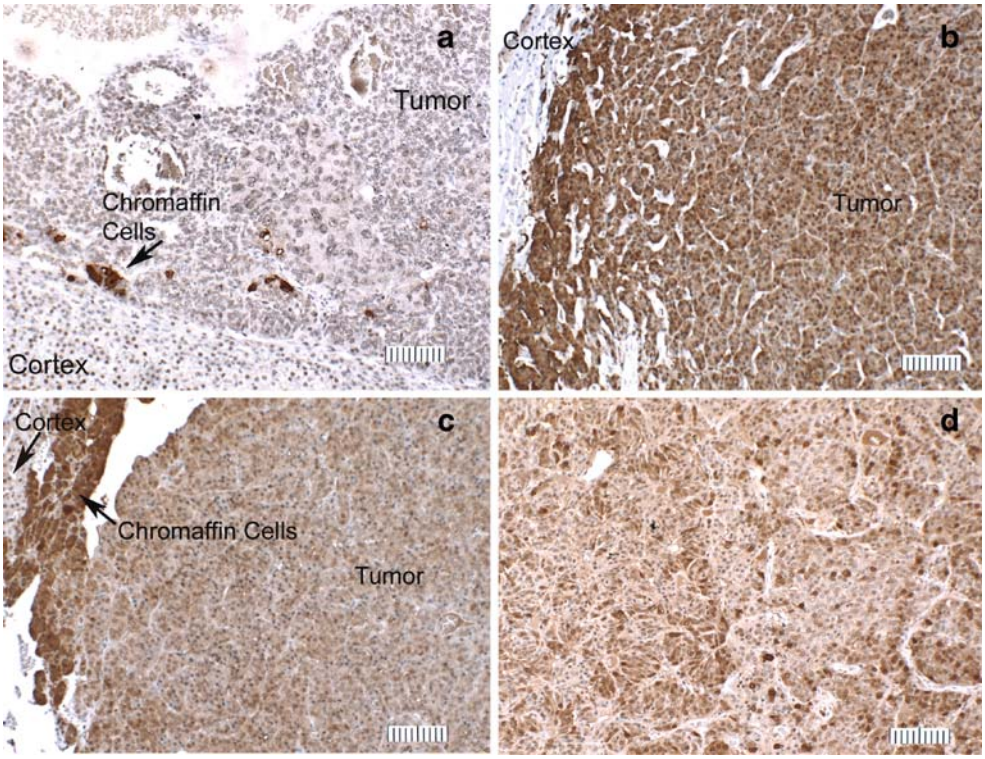

Fig. 2 Immunohistochemical patterns of PNMT immunoreactivity in rat pheochromocytomas. **a** Tumor is negative for PNMT, while residual normal chromaffin cells are strongly positive. **b** Tumor is strongly and diffusely positive. Residual normal chromaffin cells are not identifiable. **c** Tumor is diffusely positive, but staining is weak compared to residual normal chromaffin cells. **d** Tumor contains positively stained cells of variable staining intensity admixed with cells showing no immunoreactivity. Tumor in **a** was from a Long–Evans rat; **b**, **c**, and **d** from F344 rats. **d** corresponds to tumor no. 8 in Fig. 1. Sections from other tumors shown in the blot were not available for staining. *Bars*=100 μm

histochemically and conversely. However, patterns of immunohistochemical staining varied, with some tumors showing immunoreactivity in all cells and others showing admixtures of cells that were clearly positive or clearly negative (Fig. 2) Sections of the five tumors from Long–Evans hooded rats, which had been reported to contain little or no epinephrine in a small biochemical study [10], were found to be PNMT negative immunohistochemically, while

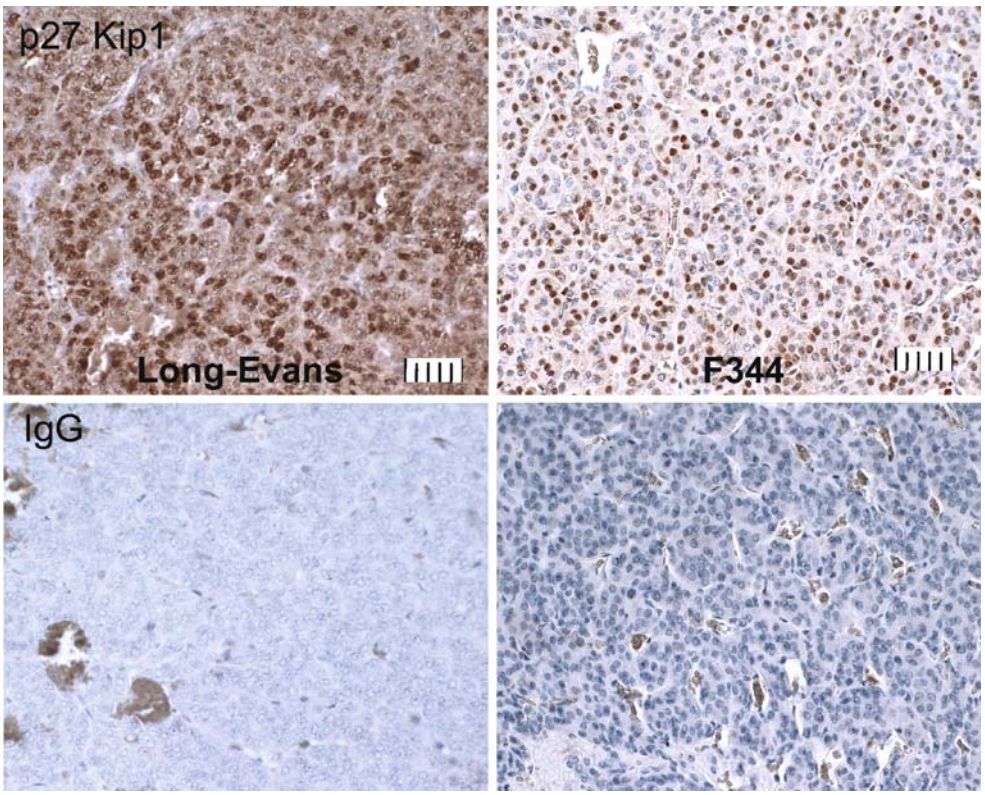

Fig. 3 Immunoreactivity for p27 ^{Kip1} in pheochromocytomas from Long–Evans and F344 rats. Nuclei in the majority of cells stain for p27 ^{Kip1} in both tumors, indicating that the Long–Evans rats did not have the *Cdkn1b* nonsense mutation recently reported to cause a multiple endocrine neoplasia syndrome in rats and humans [15]. *Bottom panels* show absence of staining in immunohistochemical controls with IgG substituted for the primary antibody. *Bars*=50 μm

normal adrenal medulla on the same slides was strongly positive (Fig. 2). All but one tumor showed nuclear staining for p27, as did normal adrenal medulla (Figs. 3 and 4). The exception was one tumor from the Long–Evans study that showed high background and absent staining of normal medulla and was therefore considered uninterpretable.

Tumor Morphology

Scanned images of hematoxylin-and-eosin-stained histologic sections of all of the tumors were examined for possible correlations of histological features with Ret and/or PNMT expression. No distinguishing features were identified. There were also no apparent morphological or phenotypic differences between control and chemically induced tumors. Occasional areas of ganglioneuroma admixed with pheochromocytoma were observed in tumors from both groups. Ganglioneuroma is known to occasionally coexist with pheochromocytomas in tumors of laboratory rats [21].

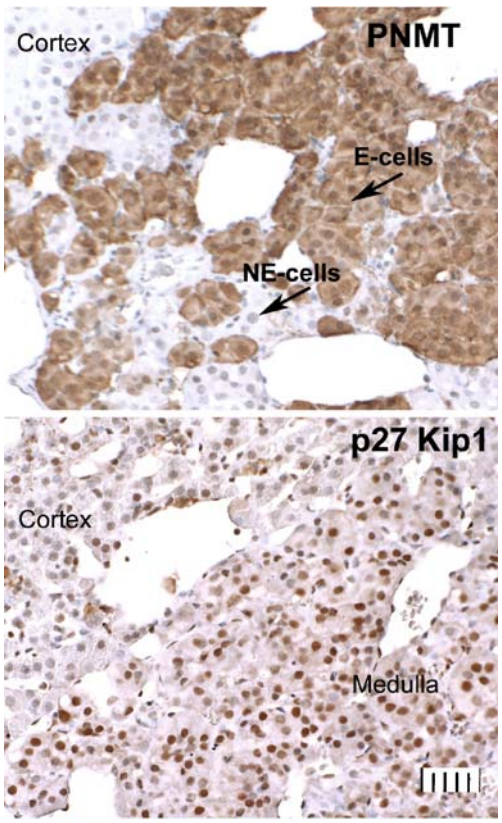

Fig. 4 Immunostaining for PNMT and p27 ^{Kip1} in normal rat adrenal. Approximately 75% of normal adrenal chromaffin cells are PNMT-positive cells that produce epinephrine (*E-cells*). PNMT-negative norepinephrine-producing cells (*NE-cells*) form small islands amid the E-cells. The majority of both cell types show nuclear staining for p27^{Kip1}. *Bar*=50 μm

Discussion

The present findings indicate that rat pheochromocytomas express an adrenergic phenotype, as defined by PNMT expression, more frequently than previously believed and that they also frequently overexpress Ret. In this study, PNMT expression was strongly associated with tumors arising in female rats, while overexpression of Ret did not show a sex predilection. The findings also show that commonly encountered rat pheochromocytomas exhibit apparently normal or increased expression of the cell cycle checkpoint protein, p27^{kip1}.

Based largely on previous immunohistochemical studies from our own laboratory, rat pheochromocytomas are believed to be almost always noradrenergic [9]. It was therefore surprising to find that a substantial percentage of spontaneous or drug-induced tumors in the present study expressed PNMT. The discrepancy with previous findings is not apparently due to the immunohistochemical technique because tumors that we reported as noradrenergic in a 1999 rat study [22] were stained with the current technique, including antigen retrieval. An alternate explanation is that the present study sampled tumors arising in a wider variety of animals, including males and females. In contrast, most of the tumors that were previously studied immunohistochemically were from males, as were the Long–Evans rat tumors [10] and the parent tumor of the PC12 cell line [23]. Both spontaneous and experimentally induced pheochromocytomas are much more common in male rats than in females, and this gender predilection has certainly affected the selection and development of experimental models [8]. The possible influence of female gender on PNMT expression in rats is intriguing, and it will be of interest to see whether the association persists in larger series. No such association is known to exist in humans. In a preliminary study, we have tested the effect of estradiol on PNMT expression in PC12 cells and found no effect (Powers, unpublished data).

The finding that rat pheochromocytomas frequently overexpress Ret similarly to many human pheochromocytomas was also somewhat surprising because PC12 cells, which are currently the most widely studied model of rat pheochromocytoma and the only rat pheochromocytoma cell line, express very low levels of endogenous Ret [12, 24]. PC12 cells are also noradrenergic, while human pheochromocytomas that overexpress RET are often adrenergic. Mouse pheochromocytomas that overexpress Ret are also frequently adrenergic [12].

Although rats frequently develop pheochromocytomas, the relevance of these tumors as a model for human disease is uncertain for several reasons. With the exception of the unusual, recently reported, *Cdkn1b* gene [15], the genetic abnormalities that lead to development of pheochromocy-

tomas in rats are unknown. In the present study, we demonstrated abundant $p27^{kip1}$ in every tumor studied, indicating that the same type of *Cdkn1b* mutation is not responsible either for the pheochromocytomas frequently encountered in toxicology studies or for the mixed MEN-like syndrome previously reported in Long–Evans rats, despite the apparent similarity of that syndrome to the syndrome associated with *Cdkn1b*.

In our previous studies, we found that in addition to being noradrenergic, rat pheochromocytomas tended to contain relatively sparse secretory granules. The combination of findings at one point led us to speculate that the tumors might arise from a noradrenergic progenitor, perhaps related to the small granule-containing cell in rat sympathetic ganglia [10] that does not exist in significant numbers in the adult human adrenal. Similarly, the associations of a noradrenergic phenotype with low Ret expression in PC12 cells and an adrenergic phenotype with Ret overexpression in human and mouse pheochromocytomas raised the possibility of a nonrandom association between Ret and the adrenergic phenotype as it might occur, for example, if adrenergic or noradrenergic pheochromocytomas arose, respectively, from Ret-positive or Ret-negative progenitors. The absence of any clear association between overexpression of Ret and expression of PNMT in this study argues against that model, at least in rats.

The biological and functional significance of Ret expression in rat pheochromocytomas is unknown. We were unable to perform mutation analyses in the present study. However, the fact that wild-type Ret is frequently overexpressed in both human and mouse pheochromocytomas, together with the absence of other stigmata of MEN2 in rats that develop pheochromocytomas, suggests that *Ret* in these animals is probably wild type. This remains to be confirmed in future investigations. It is of interest that in the normal adult, adrenal Ret is expressed at high levels in neurons, which are noradrenergic, but not in chromaffin cells [17]. However, both normal and neoplastic chromaffin cells can acquire neuron-like traits in cell culture [25] or in transplants [26]. To a lesser extent, Ret can be upregulated in normal chromaffin cells within the adrenal by intense physiological stimulation [17]. Overexpression of wild-type Ret might therefore be a reflection of phenotype plasticity and could result from the activation of signaling pathways that increase Ret expression in the normal adrenal medulla [17] or in pheochromocytoma cells [27]. In mouse pheochromocytoma cell lines that overexpress wild-type Ret, glial cell line-derived neurotrophic factor (GDNF), which is a Ret-activating ligand, consistently activates Ret signaling but evokes varying responses at the cellular level in different cell lines [12]. Some lines show proliferation arrest and process outgrowth, while others show no discernible effect. We therefore presently believe that wild-type Ret is a tumor marker but that it does not play a consistent or necessary role in tumor growth or survival.

In summary, the present results show that rat pheochromocytomas have greater phenotypic diversity and greater similarity to their human counterparts than previously believed with respect to the expression of Ret and PNMT. Studies of these tumors may contribute to the understanding of the cellular mechanisms that underlie distinctive phenotypes.

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