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Catecholamine metabolomic and secretory phenotypes in pheochromocytoma

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Abstract

Pheochromocytomas and paragangliomas (PPGLs) are highly heterogeneous tumours with variable catecholamine biochemical phenotypes and diverse hereditary backgrounds. This analysis of 18 catecholamine-related plasma and urinary biomarkers in 365 patients with and 846 subjects without PPGLs examined how catecholamine metabolomic profiles are impacted by hereditary background and relate to variable hormone secretion. Catecholamine secretion was assessed in a subgroup of 156 patients from whom tumour tissue was available for measurements of catecholamine contents. Among all analytes, the free catecholamine O-methylated metabolites measured in plasma showed the largest tumour-related increases relative to the reference group. Patients with tumours due to multiple endocrine neoplasia type 2 and neurofibromatosis type 1 (NF1) showed similar catecholamine metabolite and secretory profiles to patients with adrenaline-producing tumours and no evident hereditary background. Tumours from these three groups of patients contained higher contents of catecholamines, but secreted the hormones at lower rates compared to tumours that did not produce appreciable adrenaline, the latter including PPGLs due to *von Hippel-Lindau* and *succinate dehydrogenase* gene mutations. Large increases of plasma dopamine and its metabolites additionally characterized patients with PPGLs due to the latter mutations, whereas patients with NF1 were characterized by large increases in plasma dihydroxyphenylglycol and dihydroxyphenylacetic acid, the deaminated metabolites of noradrenaline and dopamine. This analysis establishes the utility of comprehensive catecholamine metabolite profiling for characterizing the distinct and highly diverse catecholamine metabolomic and secretory signatures among different groups of patients with PPGLs. The data further suggest developmental origins of PPGLs from different populations of chromaffin cell progenitors.

Keywords

phaeochromocytoma; paraganglioma; noradrenaline; adrenaline; dopamine; normetanephrine; metanephrine; methoxytyramine; von Hippel-Lindau syndrome; neurofibromatosis type 1; multiple endocrine neoplasia type 2; succinate dehydrogenase

Introduction

Phaeochromocytomas and paragangliomas (PPGLs) are heterogeneous tumours with highly variable signs and symptoms and diverse clinical presentations (Manger 2009). Much of the heterogeneity is due to wide ranging variations in the types and relative amounts of catecholamines produced by the tumours and differences in episodic versus continuous secretion (Eisenhofer, et al. 2001; Feldman 1981; Feldman, et al. 1979; Ito, et al. 1992). This in turn appears to reflect differences in expression of genes regulating catecholamine biosynthetic and secretory processes (Eisenhofer, et al. 2008; Isobe, et al. 2000; Timmers, et al. 2008).

Synthesis of catecholamines is primarily regulated by the cytoplasmic enzyme, tyrosine hydroxylase, which converts tyrosine to dihydroxyphenylalanine (DOPA) (Nagatsu and Stjarne 1998). DOPA is then converted to dopamine, which is translocated from the cytoplasm into catecholamine storage vesicles. Presence of dopamine- β hydroxylase within storage vesicles of noradrenergic nerves and chromaffin cells leads to conversion of dopamine to noradrenaline. In adrenal medullary chromaffin cells, noradrenaline is metabolized to adrenaline by phenylethanolamine-N-methyltransferase (PNMT). Since that enzyme is located in the cytoplasm, production of adrenaline depends on leakage of noradrenaline from storage vesicles by a continuous process that is counterbalanced by the actions of vesicular monoamine transporters to return cytoplasmic catecholamines back into storage vesicles.

Metabolism of catecholamines occurs by a plethora of pathways resulting in numerous metabolites, but primarily first occurs in the same cells where the catecholamines are synthesized (Eisenhofer, et al. 2004b). Presence of monoamine oxidase in noradrenergic neurons of the central and sympathetic nervous systems means that most noradrenaline produced at these sites is deaminated to dihydroxyphenylglycol (DHPG) following neuronal reuptake or leakage of the transmitter from storage vesicles into the neuronal cytoplasm. Additional presence of catechol-O-methyltransferase within adrenal chromaffin cells means that the noradrenaline and adrenaline produced there are respectively metabolized to normetanephrine and metanephrine; again as in noradrenergic neurons this depends on leakage of the catecholamines from storage vesicles into the cytoplasm. Metabolism of dopamine can also occur by multiple pathways leading to production of the deaminated metabolite, dihydroxyphenylacetic acid, or the O-methylated metabolite, methoxytyramine.

The present analysis utilized a dataset from a large population of patients, linked to a tumour tissue bank, to comprehensively characterize the catecholamine metabolite profiles of patients with PPGLs, and any relationships of metabolomic signatures to different catecholamine secretory phenotypes and hereditary backgrounds. A total of 18 catecholamine-related plasma and urinary biomarkers were examined in 365 patients with PPGLs and 846 subjects without PPGLs who served as reference group. The primary aim was to characterize the presence of distinct catecholamine biochemical and secretory phenotypes among PPGLs as a basis for future studies exploring the pathogenetic characteristics and developmental origins of the tumours from different populations of chromaffin cell progenitors.

Subjects and Methods

Subjects

The study involved retrospective analysis of data from 1211 subjects, including 365 patients with pathologically confirmed PPGLs and 846 subjects without PPGLs. The latter group was included for comparative purposes to establish in patients with PPGLs the relative increases of plasma and urine tumour biomarkers above normal. Subjects were investigated under multicenter clinical protocols, based mainly at the National Institutes of Health (NIH) in Bethesda (Maryland, USA), but which also included several European Centers. The latter included Radboud University Medical Center (Nijmegen, the Netherlands), the University of Florence (Florence, Italy), Gothenburg University Hospital (Gothenburg, Sweden) and Dresden University Hospital (Dresden, Germany). Written informed consent was obtained from patients enrolled into intramural review board approved studies at the NIH, which also allowed for collection of patient samples at offsite centers.

Patients with PPGLs had a mean age of 40 years (range 6 to 83 years) at initial diagnosis of tumours and included 190 males and 175 females. Adrenal and extra-adrenal locations of tumours were determined using results of imaging studies and surgical and pathological records. Among the patients with PPGLs there were 173 with clearly identified hereditary syndromes or gene mutations and 192 in whom there was no clearly identified mutation or evidence of an established hereditary syndrome. The high proportion of patients with hereditary syndromes or gene mutations largely reflects disproportionate referral of those patients to the participating specialist centers. Amongst the group of 173 patients with hereditary PPGLs, there were 66 with von Hippel-Lindau (VHL) syndrome, 38 with multiple endocrine neoplasia type 2 (MEN 2), 10 with neurofibromatosis type 1 (NF1), 59 with mutations of the *succinate dehydrogenase* (*SDH*) genes, including 48 with mutations of the type B subunit (*SDHB*) gene and 11 of the type D subunit (*SDHD*) gene. All patients with MEN 2 and NF1 and most with VHL syndrome were diagnosed with already established gene mutations or hereditary syndromes at the time of testing for catecholamine-producing tumours. However, 1 patient was identified with a *VHL* mutation, 37 with *SDHB* mutations and 8 with *SDHD* mutations as a consequence of routine testing of *RET*, *VHL*, *SDHD* and *SDHB* genes implemented after 2005.

Among the 192 patients with no evidence of an established hereditary syndrome, gene testing failed to confirm the presence of mutations of *VHL*, *RET*, *SDHD* or *SDHB* genes in genomic DNA available from 94 patients. The study did not include testing of the more recently described tumour susceptibility genes for the SDH complex assembly factor 2 and transmembrane protein 127.

The 846 subjects without PPGLs included 379 males and 467 females. Subjects had a mean age of 41 years (range 6 to 84 years) and included 175 normotensive volunteers, 94 hypertensive volunteers and 577 patients in whom testing for PPGLs was carried out and tumours were excluded by previously described criteria (Lenders, et al. 2002). The use of medications known to cause false-positive elevations of plasma or urinary catecholamines and metanephrines (e.g., tricyclic antidepressants and phenoxybenzamine) constituted additional exclusion criteria.

Collections of blood, urine and surgical specimens

Blood samples from all 1211 study participants were obtained with subjects supine for at least 20 min before blood collection. Subjects were instructed to fast and abstain from caffeinated and decaffeinated beverages overnight and avoid taking acetaminophen for 5 days before blood sampling. Samples of blood were transferred into tubes containing

heparin as anticoagulant and immediately placed on ice until centrifuged (4°C) to separate the plasma. Plasma samples were stored at –80°C until assayed.

Twenty-four hour urine samples were collected from 338 of the 365 patients with PPGLs and 513 of the 847 subjects without tumours. Samples were collected with hydrochloric or another acid as a preservative, total urine volume was determined and aliquots kept at 4°C until assayed.

Samples of tumour tissue were procured from 156 patients with PPGLs, generally within 1 hour of surgical resection of tumours. The dimensions of tumours were recorded. Small samples of each tumour (10 to 50 mg) were dissected from the mass, frozen on dry ice and stored at –80°C. As part of further processing, tissue samples were weighed frozen and then homogenized in at least 5 volumes of 0.4 M perchloric acid containing 0.5 mM EDTA. Homogenized samples were centrifuged (1500 × g for 15 min at 4°C) and supernatants collected and stored at –80°C until assayed for catecholamines.

Laboratory analyses

Plasma, urinary and tissue catecholamines (noradrenaline, adrenaline and dopamine) and plasma and urinary fractionated metanephrines (normetanephrine and metanephrine) were quantified by liquid chromatography with electrochemical detection. Concentrations of catecholamines were determined after extraction from plasma or perchloric acid tissue supernatants using alumina adsorption as described previously (Eisenhofer, et al. 1986). The assays of catecholamines in plasma also included measurements of three other catechols: 3,4-dihydroxyphenylalanine (DOPA), the precursor of dopamine and product of the rate limiting step in catecholamine biosynthesis; 3,4-dihydroxyphenylglycol (DHPG), a deaminated metabolite of noradrenaline and adrenaline produced principally within sympathetic nerves; and 3,4-dihydroxyphenylacetic acid, the deaminated metabolite of dopamine.

Plasma and urinary fractionated metanephrines (normetanephrine and metanephrine) were estimated using different liquid chromatographic methods as described elsewhere (Lenders, et al. 1993; Lenders et al. 2002). The assays in plasma also allowed determination of methoxytyramine, the O-methylated metabolite of dopamine. Assays of plasma concentrations of metanephrine, normetanephrine and methoxytyramine were principally directed to measurements of the free metabolites (i.e., free metanephrines and methoxytyramine). However, additional measurements of the much higher concentrations of deconjugated metanephrines were also carried in 192 and 789 respective subjects with and without PPGLs. These latter measurements were carried out after incubating 200 µl aliquots of plasma with sulphatase over 30 minutes for 37°C and reflect concentrations of both free and sulphate-conjugated metabolites, similar to the measurements of urinary fractionated metanephrines.

Data analyses

Differences in signal strengths of the 18 plasma and urinary catecholamine-related analytes were assessed from comparisons of their relative increases in patients with PPGLs above mean values in the reference population. The 95% confidence intervals of values in the reference group were also calculated for provision of lower and upper limits of normal, as described elsewhere (Eisenhofer, et al. 1999). Values from the reference group were used for comparisons of catecholamine-related analytes in subgroups of patients with PPGLs, as outlined below.

Patients identified with disease-causing mutations or hereditary syndromes were divided into five subgroups (VHL, MEN 2, NF1, SDHB and SDHD) according to the nature of the

syndrome or gene mutation that was detected. Patients without evidence of a hereditary syndrome or mutation were divided into three subgroups according to the noradrenergic, adrenergic or dopaminergic phenotypes of their tumours, as based on previous findings relating tumour tissue contents of the different catecholamines to increases in plasma concentrations of their respective O-methylated metabolites (Eisenhofer, et al. 2005b). For this, tumour-derived increments of plasma normetanephrine, metanephrine and methoxytyramine were established by subtracting the concentration of each metabolite in each patient with a PPGL from the mean concentration in the reference group. Noradrenergic tumours were defined as those with predominant increases of only normetanephrine, accompanied by either normal plasma concentrations of metanephrine and methoxytyramine (below the upper reference intervals) or by increases of less than 5% for metanephrine and 10% for methoxytyramine relative to the sum of increments for all 3 metabolites. Conversely adrenergic and dopaminergic tumours were defined as those characterized by respective increases of plasma metanephrine and methoxytyramine above the upper reference limits and associated increments, relative to the combined increments of all 3 metabolites, of larger than 5% for metanephrine and 10% for methoxytyramine.

For estimations of tumour-derived catecholamine secretion into plasma or excretion into urine, differences in plasma concentrations (nmol/L) or urinary outputs ($\mu\text{mol/day}$) of catecholamines in patients with PPGLs compared with mean values in the reference population of subjects without tumours provided estimates of increases in the amines due to tumours. Rates of catecholamine secretion from tumours into plasma were estimated using the formula, $S = P \times C \times 1.44$, where S is the rate of catecholamine secretion ($\mu\text{mol/day}$), P is the plasma concentration of catecholamines due to the tumour (nmol/L), C is the circulatory clearance of catecholamines from plasma (L/min) and where the value, 1.440, was used to convert secretion rates of nmol/min to $\mu\text{mol/day}$, the same units as for urine. The formula was based on that described elsewhere (Eisenhofer et al. 2008).

Rates of catecholamine secretion into plasma or excretion into urine ($\mu\text{mol/day}$) were divided by estimates of tumour volume to normalize for differences in tumour size and derive final rates in units of $\mu\text{mol/day}$ per cubic cm of tumour. Volumes of tumours (V) in cubic cm were estimated using the formula for the volume of a sphere, $V = 4/3 \pi r^3$, where r , the radius in cm, was derived from estimated mean diameters (the latter calculated from the cubed roots of rectangular volumes).

Rate constants for catecholamine secretion into plasma or excretion into urine (day^{-1}) — representing the proportions of total catecholamines in a tumour secreted into plasma or excreted into urine over a day — were estimated by dividing rates of catecholamine secretion into plasma or excretion into urine ($\mu\text{mol/day}$) by total tumour catecholamine contents (μmol). Total tumour catecholamine contents were estimated from the product of tissue catecholamine concentrations and tumour mass (the latter derived from tumour volume, assuming a specific gravity of 1.0).

Statistics

Due to the skewed distributions of plasma concentrations and urinary outputs of catecholamines and their metabolites, statistical significance of differences in neurochemical data was determined in all cases after logarithmic transformation. Differences between comparisons of two groups was determined by Student's t-test and between comparisons of multiple groups by one-way or two-way ANOVA depending on respective absence or presence of more than one independent variable. Post-hoc tests associated with multiple comparisons included Dunnett's test for comparisons against a single reference group or the Tukey-Kramer test for comparisons amongst all groups.

Results

Plasma and urine catecholamine metabolomic profiles

Increases in plasma concentrations and urinary outputs of the three endogenous catecholamines, their O-methylated and deaminated metabolites and their amino acid precursor, DOPA, showed considerable variability among the 365 patients with PPGLs relative to the reference population of 846 subjects (Fig. 1). Among the principal metabolites of noradrenaline, DHPG in plasma and VMA in urine showed the lowest signal strengths in patients with PPGLs. Plasma free normetanephrine showed the highest signal strength amongst all 18 catechol-related analytes profiled, with a 12.2-fold increase above the reference population that significantly ($P<0.0004$) surpassed all other analytes, including deconjugated normetanephrine in plasma (7.4-fold increase) and urine (6.7-fold increase).

Among the O-methylated metabolites of adrenaline, there were no differences in signal strengths of free or deconjugated metanephrine in urine or plasma, but relative increases of these metabolites were larger ($P<0.05$) than those for plasma and urinary adrenaline (Fig. 1). Among the dopamine-related analytes, plasma concentrations of free methoxytyramine showed the largest signal with 3.3-fold increases above the reference population, surpassing ($P<0.006$) all other dopamine-related analytes. Plasma DOPA, DOPAC and urinary dopamine were increased by only 10 to 38% above mean levels of the reference population.

Distinct patterns in the profiles of the eighteen catechol-related analytes emerged after patients with PPGLs were grouped according to hereditary syndromes or, in patients without hereditary syndromes, according to catecholamine phenotype (Table 1). Among the latter patients, there were 87 with predominantly noradrenaline-producing tumours (noradrenergic phenotype), 95 with tumours that produced significant amounts of adrenaline (adrenergic phenotype) and 10 with tumours characterized by significant dopamine production (dopaminergic phenotype).

The clearest pattern to emerge was a divergence in increases of plasma concentrations and urinary outputs of adrenaline and its O-methylated metabolite, metanephrine, measured in both free and deconjugated forms (Table 1). Specifically, patients with *VHL*, *SDHB* and *SDHD* mutations showed no significant increases of plasma or urinary adrenaline and free or deconjugated metanephrine above reference levels. In contrast, patients with MEN 2 and NF1 showed highly significant ($P<0.0001$) 7- to 31-fold increases of all adrenaline-related analytes, a pattern similar to that in patients with adrenergic tumours and no clear hereditary syndrome.

Patients with NF1 were further distinguished from MEN 2 patients and other patient groups by higher ($P<0.05$) plasma concentrations of DHPG (Table 1). Plasma concentrations of DOPAC were also higher ($P<0.05$) in patients with NF1 than in patients with MEN 2 and VHL syndrome.

Larger ($P<0.0001$) increases of plasma free normetanephrine relative to noradrenaline in patients with adrenergic than noradrenergic or dopaminergic tumours represented another pattern in neurochemical profiles (Table 1). More specifically, increases of plasma normetanephrine above reference were 4.3- to 7.8 fold larger than those of noradrenaline in all patients with adrenaline-producing tumours, including those with MEN 2 and NF1. This contrasted with patients who had noradrenergic or dopaminergic tumours, including patients with *VHL*, *SDHB* and *SDHD* mutations, in whom increases in plasma free normetanephrine were only 1.8- to 3.0-fold larger than those of noradrenaline. Thus, regardless of the presence or absence of a mutation, patients with adrenaline-producing tumours had larger

increases of plasma free normetanephrine relative to noradrenaline than patients with tumours that lacked significant adrenaline production.

Distinctly larger increases of dopamine-related analytes in patients with tumours due to *SDHB* and *SDHD* mutations compared to other mutations represented another clear difference in profiles of catechol-related analytes among patients groups (Table 1). In particular, plasma concentrations of dopamine and free methoxytyramine were more than 90-fold higher ($P<0.0001$) than reference in patients with *SDHB* mutations and more than 70-fold higher ($P<0.001$) in patients with *SDHD* mutations. In contrast, these analytes showed less than 4-fold increases above reference in *VHL*, *MEN 2* and *NF1* patients. Also in striking contrast to the more than 70-fold increases in plasma free methoxytyramine and dopamine, urinary outputs dopamine in patients with *SDHB* and *SDHD* mutations were respectively increased by only 2.9- and 3.3-fold above reference.

Tumour tissue catecholamine contents

Tumour tissue concentrations of catecholamines showed considerable variability among the different groups of patients with and without established disease causing mutations (Table 2). The presence of markedly higher tumour tissue concentrations of adrenaline in patients with *MEN 2* and *NF1* than in those with mutations of *VHL*, *SDHB* and *SDHD* genes represented the clearest distinguishing feature among patients with hereditary PPGLs.

Tumour tissue concentrations of noradrenaline also showed considerable differences among groups, with lowest levels observed in patients with *SDHB* mutations and, at the other extreme, close to 6-fold higher ($P<0.05$) levels in patients with *MEN 2* (Table 2). Tumour concentrations of noradrenaline in *MEN 2* patients were also significantly ($P<0.05$) higher than in patients with noradrenergic sporadic tumours. Total tissue concentrations of catecholamines (sum of dopamine, noradrenaline and adrenaline) were consequently highest in adrenaline-producing sporadic and hereditary tumours, and lowest in all other groups of tumours (Fig. 2A).

Catecholamine secretory phenotypes

Rates of tumour-derived catecholamine secretion into plasma (Fig. 2B) or excretion into urine (Fig. 2C), normalized to tumour volume, showed a reciprocal pattern compared to that for the differences of tumour tissue catecholamines among the groups (Fig. 2A). Specifically, while tissue concentrations of catecholamines were lowest in tumours with a noradrenergic or dopaminergic phenotype and highest in those with an adrenergic phenotype, the former tumours were characterized by high rates and the latter by low rates of catecholamine secretion into plasma and excretion into urine. Patients with *VHL* syndrome or with noradrenergic tumours and no identified hereditary syndrome showed particularly high rates of tumoural catecholamine secretion, averaging 4.8- to 6.9-fold higher than in patients with *MEN 2* and *NF1* or with adrenergic tumours and no identified hereditary syndrome.

The differences in tumour-derived catecholamine secretion and excretion among the different patient groups were particularly pronounced when assessed as rate constants for catecholamine secretion into plasma (Fig. 2D) or excretion into urine (Fig. 2E). The rate constants for catecholamine secretion into plasma indicated that adrenaline-producing sporadic and hereditary tumours released only 2% to 5% of their tumour tissue contents of catecholamines into the bloodstream per day, compared to 57% for sporadic noradrenergic tumours and 34%, 46% and 15% respectively for tumours from patients with *VHL*, *SDHB* and *SDHD* gene mutations. Similarly, only between 0.1% and 0.4% of the catecholamine contents of adrenaline-producing sporadic and hereditary tumours were excreted into urine

per day, compared to 3.0% for sporadic noradrenergic tumours and between 1.2% and 4.9% for tumours from patients with *VHL*, *SDHB* and *SDHD* gene mutations.

Discussion

This study involving a large cohort of well-characterized patients with PPGLs provides a comprehensive dataset about relative increases in catecholamines and catecholamine metabolites in patients with the tumours and outlines novel findings concerning variations in the catecholamine metabolomic and secretory phenotypes among different subgroups of patients. These data have relevance to emerging concepts concerning the highly heterogeneous nature of PPGLs, how this heterogeneity relates to underlying germ-line mutations of tumour susceptibility genes and how this in turn explains distinct differences in gene expression profiles and pathways of development of tumours from different chromaffin cell progenitors.

Catecholamine metabolomic profiles explained

The profiling of catecholamine-related analytes in plasma and urine establishes that among all precursor amines and metabolites examined, the free O-methylated metabolites provide higher signal strengths for indicating the presence of PPGLs than their precursor catecholamines or corresponding deaminated and sulphate-conjugated metabolites. The higher signal strengths of the O-methylated metabolites than of their monoamine precursors has been amply demonstrated (Grossman, et al. 2006), and is established to reflect continuous production of O-methylated metabolites within chromaffin tumour cells by processes that are independent of variations in exocytotic catecholamine secretion (Eisenhofer, et al. 1998). The particularly low signal strength of DHPG is in agreement with previous findings (Brown 1984) and reflects the substantial and almost exclusive production of this metabolite within sympathetic nerves (Figure 3), which considerably dilutes any additional contribution from chromaffin tumour cells. Formation of VMA within the liver, mainly after extraneuronal O-methylation of the DHPG formed within sympathetic neurons (Eisenhofer et al. 2004b), further explains the relatively low diagnostic signal strength of this end product of noradrenaline and adrenaline metabolism.

The higher diagnostic signal strengths of plasma free than of plasma and urinary deconjugated normetanephrine and methoxytyramine are also explained by different sources of the free compared to the sulphate-conjugated metabolites (Figure 3). Sulphate conjugation occurs principally in gastrointestinal tissues as a mechanism for inactivating both dietary-derived monoamines and the substantial amounts of endogenous dopamine and noradrenaline produced by mesenteric organ catecholamine systems (Eisenhofer et al. 2004b). The contributions of these sources to levels of sulphate-conjugated normetanephrine and methoxytyramine thereby dilute the signals of the sulphate-conjugated metabolites derived from the free metabolites produced within chromaffin tumour cells.

Because almost all adrenaline is produced within adrenal chromaffin cells, there is little influence of other sources of that catecholamine to dilute any diagnostic signal of sulphate-conjugated metanephrine relative to its free metanephrine precursor; thus, in contrast to normetanephrine and methoxytyramine, both free and deconjugated metanephrine have similar diagnostic signal strengths. Nevertheless, since most of the diagnostic signal strength of the O-methylated metabolites reflects that of normetanephrine, the free metabolites provide more sensitive and specific biomarkers for PPGLs compared to the deconjugated metanephrines (Lenders et al. 2002; Unger, et al. 2009). They are also less susceptible to dietary influences (de Jong, et al. 2009).

The much lower signal strength of urinary dopamine compared to plasma dopamine and plasma free and deconjugated methoxytyramine is explained by the more than 90% of dopamine in urine that is derived from renal extraction and decarboxylation of circulating DOPA (Brown and Allison 1981). This large contribution of plasma DOPA to urinary dopamine considerably dilutes any signal of tumour-derived urinary dopamine. Thus, compared to plasma measurements of dopamine and methoxytyramine, measurements of urinary dopamine provide an insensitive biomarker of tumour dopamine production (Eisenhofer, et al. 2005a).

Catecholamine metabolomic phenotypes

Our observations of distinct patterns in catecholamine metabolomic profiles among different groups of patients with hereditary PPGLs extend and bring together previous isolated observations about different catecholamine phenotypes and gene expression profiles. In particular, the present data showing distinct catecholamine metabolomic profiles in tumours from NF1 and MEN 2 patients, compared to patients with mutations of *VHL*, *SDHB* and *SDHD* genes, are consistent with patterns in gene expression profiles observed by others (Dahia, et al. 2005; Favier, et al. 2009). Using unsupervised hierarchical cluster analysis, these groups both established the presence of two dominant expression clusters, one that included tumours from NF1 and MEN 2 patients and the other tumours from patients with mutations of *VHL*, *SDHB* and *SDHD* genes. The differences in gene expression indicated two pathways of tumorigenesis: one pathway in *VHL*, *SDHB* and *SDHD* related tumours involved activation of hypoxia-angiogenesis related genes and the other in tumours from MEN 2 and NF1 patients involving increased kinase signaling. The present data additionally suggest different origins of the two cluster groups from adrenergic and noradrenergic chromaffin cell precursors, or alternatively, downstream effects of the differentially activated signaling pathways on expression of PNMT, the enzyme that converts noradrenaline to adrenaline.

Interestingly, while tumours in NF1 and MEN 2 patients exhibited similar adrenergic phenotypes, large increases of both plasma DHPG and DOPAC provided an additional feature that distinguished tumours in NF1 patients from those of other groups. The substantial increases of these deaminated metabolites of noradrenaline and dopamine suggest higher activity of monoamine oxidase and a more neuronal-like metabolizing phenotype in NF1-associated tumours than in other PPGLs.

Furthermore, while tumours in patients with mutations of *VHL* and *SDH* genes all exhibited negligible adrenaline production, large increases of dopamine and dopamine-related metabolites in patients with *SDH* mutations represented an additional characteristic that distinguished this group from patients with tumours due to *VHL* mutations. Thus, while transcriptomic profiling studies indicate that tumours with *SDH* and *VHL* mutations are closely linked (Dahia et al. 2005), the present catecholamine metabolomic profiling data clearly indicate different dopaminergic and noradrenergic neurochemical signatures among the two groups. These findings also agree with more recent transcriptomic profiling studies that although confirming the presence of two distinct cluster groups, also revealed further differences in gene expression between tumours due to *VHL* and *SDH* mutations (Favier et al. 2009). Other studies have established contrasting clinical manifestations of *SDHB* and *VHL* associated chromaffin cell tumours (Srirangalingam, et al. 2009). As further discussed below, such differences in clinical characteristics may relate to differences in catecholamine metabolomic and secretory phenotypes observed here.

Catecholamine secretory phenotypes

In addition to establishing distinct catecholamine metabolomic phenotypes, this study also establishes for the first time that the various groups of patients with adrenergic versus noradrenergic or dopaminergic tumours show distinct catecholamine secretory phenotypes. More specifically adrenaline-producing tumours contain higher concentrations of catecholamines yet show lower rates catecholamine secretion into plasma and excretion into urine compared to tumours that do not produce appreciable adrenaline.

Differences in secretory characteristics and expression of secretory pathway components in different populations of adrenergic and noradrenergic chromaffin cells are well established (Aunis and Langley 1999; Langley and Grant 1995; Marley and Livett 1987; Teraoka, et al. 1993). In particular, fractionation of bovine adrenal medullary cells into two populations of low density mainly noradrenergic and high density mainly adrenergic chromaffin cells revealed that the noradrenergic cells released a higher percentage of their catecholamine contents than adrenergic cells (Krause, et al. 1996). Similarly in the present study, noradrenergic tumours released between 15% and 57% of their catecholamine stores each day, compared to less than 5% for adrenaline-producing tumours.

The divergent lower tissue concentrations but higher rates of catecholamine secretion in noradrenergic than adrenergic PPGLs extend findings of our previous studies focusing on tumours in MEN 2 and VHL syndrome (Eisenhofer et al. 2008; Eisenhofer et al. 2001). These earlier studies showed that although tumours in VHL syndrome secrete noradrenaline at higher more continuous rates than those in patients with MEN 2, the latter tumours are more easily provoked to secrete both noradrenaline and adrenaline in episodic bursts with a resulting more symptomatic clinical presentation. These differences in secretory profiles reflect extensive differences in expression of numerous genes encoding multiple components of the regulated secretory pathway, including enzymes regulating transmitter synthesis, vesicular proteins and their processing enzymes, exocytotic machinery components as well as receptors and other signal transduction factors responsible for excitation-secretion coupling (Eisenhofer et al. 2008). In general, noradrenergic tumours due to VHL gene mutations are characterized by immature constitutive secretory pathways, whereas mature regulated secretory pathways typical of more fully differentiated adrenal medullary chromaffin cells characterize adrenergic tumours in MEN 2.

Similar differences in expression of secretory pathway components also likely extend to and explain the differences in tumour tissue contents and secretion of catecholamines among the various groups of patients of this study. The lower rates of catecholamine secretion from adrenergic than noradrenergic or dopaminergic tumours furthermore clarify other differences in the clinical presentation of the different groups. In particular, the larger relative increases of plasma normetanephrine than noradrenaline in patients with adrenaline-producing tumours than other groups reflect the lower rates of hormonal secretion in the former than latter groups. This also explains the strikingly much higher diagnostic sensitivity of metanephrines than catecholamines for detection of adrenaline- than noradrenaline-producing PPGLs (Eisenhofer et al. 2005b).

Perspective

Unlike the adrenal medulla of some animal species that contain separate populations of adrenergic and noradrenergic chromaffin cells, the adult human adrenal medulla is largely comprised of a single population of PNMT positive adrenergic cells (Cleary, et al. 2005). Although this observation argues against development of noradrenergic and adrenergic adrenal phaeochromocytomas from separate populations of chromaffin cells, other studies

now suggest that at least some chromaffin cell tumours develop from failure of neuronal apoptosis during embryonic development (Lee, et al. 2005; Tischler 2006).

Of relevance to the above studies, PPGLs with a noradrenergic phenotype over-express the gene for hypoxia-inducible transcription factor (HIF-2 α) (Eisenhofer, et al. 2004a), a transcription factor with a central role in the development of PPGLs in patients with VHL and SDH mutations (Favier et al. 2009; Pollard, et al. 2006). Of further relevance, an increasing body of evidence indicates that expression of HIF-2 α and related genes are crucial for the development of embryonic tyrosine hydroxylase expressing sympathoadrenal progenitor cells (Bishop, et al. 2008; Brown, et al. 2009; Favier, et al. 1999; Tian, et al. 1998). Blocking this expression leads to impaired development of these cells and reduced catecholamine synthesis, presumably through increased apoptosis of tyrosine hydroxylase expressing noradrenaline-producing chromaffin cells.

The above observations conversely also explain susceptibility of immature chromaffin progenitor cells to the tumourigenic influences of mutations that further increase expression of HIF-2 α . In such exposed progenitor cells, failure of developmental culling and arrested differentiation may be expected to lead to development of PPGLs with immature dopaminergic or noradrenergic catecholamine phenotypes. In contrast, development of tumours with a mature adrenergic phenotype would only be expected following migration of neural crest chromaffin progenitors into the adrenal enlargen, only after which they can be induced by locally-produced steroids to express PNMT (Adams and Bronner-Fraser 2009). This concept is also consistent with other findings that adrenaline-producing tumours present at later ages than noradrenergic PPGLs (Eisenhofer et al., unpublished observations).

In summary, previous findings of global differences in gene expression profiles among different groups of hereditary and sporadic PPGLs and the present findings of associated patterns in catecholamine metabolomic and secretory profiles together support the likelihood that many of the differences between the various groups of tumours reflect distinct origins from immature noradrenergic or dopaminergic chromaffin cell progenitors compared to the more highly differentiated adrenergic chromaffin cells of the adult adrenal medulla. The distinct catecholamine metabolomic and secretory signatures of different groups of PPGLs provides a novel framework for future studies exploring the pathogenetic developmental origins of these tumours from different populations of chromaffin cell progenitors.

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Figure 1.

Plasma and urinary catecholamine metabolomic profiles in patients with PPGLs relative to reference. Data are shown as fold-increases (means $\pm$ SEM) of values in patients with PPGLs above mean values for the reference population. Data for the catecholamines, including noradrenaline (NA), adrenaline (A) and dopamine (DA) are shown by the empty bars ($\square$); for the free O-methylated catecholamine metabolites, including normetanephrine (f-NMN), metanephrine (f-MN) and methoxytyramine (f-MTY), by the black bars ($\blacksquare$); for the deconjugated O-methylated catecholamine metabolites, including deconjugated normetanephrine (d-NMN), deconjugated metanephrine (d-MN) and deconjugated methoxytyramine (d-MTY) by the dark grey bars ($\blacksquare$); for the deaminated catecholamine metabolites, including dihydroxyphenylglycol (DHPG), vanillylmandelic acid (VMA) and dihydroxyphenylacetic acid (DOPAC), by the medium grey bars ($\blacksquare$); and for the amino

acid precursor of the catecholamines, dihydroxyphenylalanine (DOPA), by the light grey bar (■).

Figure 2.

Bar graphs showing tumour contents of catecholamines (panel A), rates of tumour-derived catecholamine secretion into plasma (panel B) and excretion into urine (panel C), and rate constants for tumour-derived catecholamine secretion into plasma (panel D) and excretion into urine (panel E). Data are shown for tumours from patients with VHL syndrome (n=44), MEN 2 (n=32), NF1 (n=6) and with mutations of SDHB (n=11) and SDHD (n=3) genes. Data are also shown for noradrenergic (NA, n=26) and adrenergic (A, n=34) tumours from patients without evidence of an established hereditary syndrome or gene mutation. Different alphabetic characters indicate significant differences ($P < 0.05$) between groups, while presence of identical characters indicates lack of a significant difference.

Figure 3.

Neuronal, extraneuronal and adrenal pathways of metabolism for noradrenaline and adrenaline. Abbreviations: NA, noradrenaline; A, adrenaline; DHPG, dihydroxyphenylglycol; MN, metanephrine; NMN, normetanephrine; MHPG, methoxyhydroxyphenylglycol; VMA, vanillylmandelic acid; MHPG-SO₄, methoxyhydroxyphenylglycol sulphate; NMN-SO₄, normetanephrine sulphate; MN-SO₄, metanephrine sulphate; MAO, monoamine oxidase; COMT, catechol-O-methyltransferase; ADH, alcohol dehydrogenase; SULT1A3, sulfotransferase type 1A3.

Table 1

Plasma and urine catecholamine metabolomic profiles in patients with PPGLs according to presence or absence of an established mutation or hereditary syndrome

Analyte	Units	Reference population		Established mutation or hereditary syndrome						No established mutation or hereditary syndrome*			
		No tumor (n = 846)	95% CI	VHL (n = 66)	MEN 2 (n = 38)	NF 1 (n = 10)	SDHB (n = 48)	SDHD (n = 11)	NA (n = 87)	A (n = 95)	DA (n = 10)		
		Mean±SE		Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE		
Noradrenaline & metabolites													
Plasma NA	nmol/L	1.70 ± 0.03	(0.51–4.18)	8.64 ± 1.50	4.19 ± 0.64	7.35 ± 1.21	26.50 ± 5.04	9.20 ± 2.61	23.49 ± 3.10	9.90 ± 1.54	41.01 ± 16.48		
Plasma F-MIN	nmol/L	0.33 ± 0.01	(0.11–0.74)	4.38 ± 1.09	6.34 ± 1.58	6.13 ± 1.97	13.15 ± 2.88	3.37 ± 0.90	14.18 ± 2.69	9.74 ± 1.59	9.92 ± 3.80		
Plasma d-MIN	nmol/L	11.92 ± 0.25	(3.85–28.12)	87.57 ± 21.61	132.20 ± 45.78	117.51 ± 62.16	281.15 ± 109.63	62.94 ± 33.62	304.92 ± 66.01	209.42 ± 61.38	135.60 ± 36.67		
Plasma DHPG	nmol/L	5.12 ± 0.06	(2.69–8.88)	6.59 ± 0.29	8.53 ± 0.94	18.41 ± 3.83	10.16 ± 1.00	9.31 ± 1.07	10.21 ± 0.87	12.07 ± 1.62	10.89 ± 2.37		
Urine NA	μmol/day	0.25 ± 0.01	(0.08–0.61)	1.46 ± 0.23	0.96 ± 0.19	1.54 ± 0.43	3.50 ± 0.75	2.08 ± 1.12	4.20 ± 0.67	1.92 ± 0.33	4.80 ± 1.82		
Urine d-MIN	μmol/day	1.61 ± 0.05	(0.45–4.14)	8.26 ± 1.81	12.45 ± 2.81	14.65 ± 4.85	32.40 ± 6.53	10.85 ± 4.20	32.25 ± 5.91	24.33 ± 5.14	27.86 ± 9.42		
Urine VMA	μmol/day	19.43 ± 0.44	(7.06–43.39)	41.96 ± 4.43	70.69 ± 13.48	126.96 ± 4.80	81.01 ± 15.09	42.53 ± 10.12	112.96 ± 15.51	108.38 ± 16.96	112.39 ± 33.59		
Adrenaline & metabolites													
Plasma A	nmol/L	0.15 ± 0.01	(0.01–0.67)	0.17 ± 0.03	0.97 ± 0.22	1.77 ± 0.71	0.21 ± 0.04	0.20 ± 0.07	0.23 ± 0.03	2.15 ± 0.32	0.34 ± 0.17		
Plasma F-MIN	nmol/L	0.15 ± 0.00	(0.04–0.45)	0.16 ± 0.01	4.54 ± 0.75	3.35 ± 0.88	0.21 ± 0.02	0.14 ± 0.03	0.40 ± 0.09	5.71 ± 0.70	0.19 ± 0.05		
Plasma d-MIN	nmol/L	4.58 ± 0.09	(1.00–14.90)	5.60 ± 0.73	111.50 ± 22.37	78.97 ± 28.67	5.86 ± 0.84	4.30 ± 1.02	13.38 ± 2.72	222.92 ± 43.15	5.29 ± 0.86		
Urine A	μmol/day	0.02 ± 0.00	(0.01–0.09)	0.03 ± 0.00	0.46 ± 0.10	0.69 ± 0.27	0.02 ± 0.00	0.01 ± 0.00	0.05 ± 0.01	0.85 ± 0.16	0.03 ± 0.01		
Urine d-MIN	μmol/day	0.49 ± 0.02	(0.09–1.56)	0.52 ± 0.04	15.28 ± 3.45	14.77 ± 4.09	0.67 ± 0.06	0.55 ± 0.14	0.98 ± 0.24	27.75 ± 7.89	1.07 ± 0.44		
Dopamine, DOPA & metabolites													
Plasma DA	nmol/L	0.08 ± 0.00	(0.01–0.41)	0.17 ± 0.05	0.15 ± 0.02	0.24 ± 0.04	10.39 ± 8.06	5.84 ± 4.87	0.33 ± 0.06	0.17 ± 0.02	36.21 ± 18.31		
Plasma F-MTY	nmol/L	0.03 ± 0.00	(0.00–0.11)	0.06 ± 0.01	0.08 ± 0.02	0.10 ± 0.03	2.72 ± 1.56	2.12 ± 1.32	0.30 ± 0.08	0.18 ± 0.03	15.82 ± 7.40		
Plasma d-MTY	nmol/L	1.75 ± 0.07	(0.20–7.41)	3.62 ± 0.77	5.95 ± 2.12	2.15 ± 1.67	86.57 ± 73.36	6.39 ± 4.70	23.20 ± 9.09	7.91 ± 1.77	322.36 ± 320.54		
Plasma DOPAC	nmol/L	8.54 ± 0.17	(3.78–16.59)	8.51 ± 0.44	8.96 ± 0.74	21.71 ± 8.79	38.72 ± 27.27	13.53 ± 4.06	9.98 ± 0.68	11.78 ± 1.32	45.95 ± 26.32		
Plasma DOPA	nmol/L	8.16 ± 0.09	(4.98–12.63)	8.00 ± 0.24	9.69 ± 0.57	20.60 ± 6.49	16.64 ± 5.15	11.40 ± 1.67	13.85 ± 1.99	13.89 ± 1.43	26.78 ± 11.84		
Urine DA	μmol/day	1.37 ± 0.10	(0.39–3.66)	1.55 ± 0.08	2.04 ± 0.17	3.64 ± 1.47	3.91 ± 1.16	4.57 ± 2.39	2.44 ± 0.29	2.13 ± 0.19	11.46 ± 4.56		

* Patients without an established mutation were divided into three groups according to whether their tumors produced predominantly noradrenaline (NA) or significant amounts of adrenaline (A) or dopamine (DA).

The data bounded by grey-shaded and empty boxes highlight differences in patterns of relative increases above reference values for plasma NA and f-NMN, whereas the data depicted with light and dark grey backgrounds in other areas of the table serve to highlight divergent patterns of increases above reference values for adrenaline or dopamine and their metabolites among the different groups of patients

Abbreviations: NA; noradrenaline; f-NMN, free normetanephrine; d-NMN, deconjugated normetanephrine; DHPG, 3,4-dihydroxyphenylglycol; VMA, vanillylmandelic acid; A, adrenaline; f-MN, free metanephrine; d-MN, deconjugated metanephrine; DA, dopamine; f-MTY, free methoxytyramine; d-MTY, deconjugated methoxytyramine; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPA, 3,4-dihydroxyphenylalanine

Tumour locations and tissue concentrations (nmol per cubic cm tumour) of catecholamines according to presence or absence of an established mutation or hereditary syndrome

Table 2

	Established mutation or hereditary syndrome					No established mutation or hereditary syndrome [*]		
	VHL	MEN 2	NF 1	SDHB	SDHD	NA	A	A
N	44	32	6	11	3	26	34	
Tumor location [†] A/E/B	40/2/2	32/0/0	6/0/0	1/9/1	3/0/0	18/7/1	33/1/0	
Noradrenaline	17687 ± 2122	30868 ± 4522	17283 ± 6728	5365 ± 1948	11963 ± 5926	10646 ± 2239	21914 ± 3953	
Adrenaline	309 ± 58	31604 ± 4689	13742 ± 5613	32 ± 10	65 ± 60	176 ± 57	20194 ± 3951	
Dopamine	61 ± 6	171 ± 24	228 ± 57	706 ± 366	43 ± 15	84 ± 22	183 ± 30	

^{*} Patients without an established mutation were divided into two groups according to whether or not their tumors contained predominantly noradrenaline (NA) or significant amounts of adrenaline (A)

[†] Tumor locations indicate adrenal (A), extradrenal (E) or both adrenal and extra-adrenal (B) tumors at surgical resection.