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Genotype-Specific Abnormalities in Mitochondrial Function Associate with Distinct Profiles of Energy Metabolism and Catecholamine Content in Pheochromocytoma and Paraganglioma

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Abstract

Purpose: Pheochromocytomas and paragangliomas (PGL) are neuroendocrine tumors of sympathetic and parasympathetic paraganglia. This study investigated the relationships between genotype-specific differences in mitochondrial function and catecholamine content in PGL tumors.

Experimental Design: Respiratory chain enzyme assays and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy at 500 MHz were conducted on homogenates of 35 sporadic PGLs and 59 PGLs from patients with hereditary mutations in succinate dehydrogenase subunits B and D (*SDHB*, *SDHD*), succinate dehydrogenase assembly factor 2, von Hippel-Lindau (*VHL*), rearranged during transfection (*RET*), neurofibromatosis type 1 (*NF1*), and myc-associated factor X.

Results: In SDHx-related PGLs, a significant decrease in complex II activity ($P < 0.0001$) and a significant increase in complex I, III, and IV enzyme activities were observed when compared to sporadic, RET, and NF1 tumors. Also, a significant increase in citrate synthase ($P < 0.0001$) enzyme activity was observed in SDHx-related PGLs when compared to sporadic-, VHL-, RET-, and NF1-related tumors. An increase in succinate accumulation ($P < 0.001$) and decrease in ATP/ADP/AMP accumulation ($P < 0.001$) was observed when compared to sporadic PGLs and PGLs of other genotypes. Positive correlations ($P < 0.01$) were observed between respiratory chain complex II activity and total catecholamine content and ATP/ADP/AMP and total catecholamine contents in tumor tissues.

Conclusions: This study for the first time establishes a relationship between determinants of energy metabolism, like activity of respiratory chain enzyme complex II, ATP/ADP/AMP content, and catecholamine content in PGL tumors. Also, this study for the first time successfully uses NMR spectroscopy to detect catecholamines in PGL tumors and provides *ex vivo* evidence for the accumulation of succinate in PGL tumors with an SDHx mutation. *Clin Cancer Res; 19(14); 3787–95. ©2013 AACR.*

Introduction

Pheochromocytomas and paragangliomas (PGL) are neuroendocrine tumors of sympathetic and parasympathetic paraganglia. PGLs of sympathetic origin (adrenal medulla and extra adrenal sympathetic tissue of abdomen, pelvis, and chest) usually produce catecholamines, whereas the tumors of parasympathetic origin (head and neck PGLs) usually do not produce significant amounts of catecholamines (1). At least 30% to 35% of the PGLs are caused by germline mutations of 10 identified tumor susceptibility genes (2). These include *VHL* (von Hippel-Lindau), *RET* (rearranged during transfection), *NF1* (neurofibromatosis type 1), *SDHA/B/C/D* (succinate dehydrogenase subunits A, B, C, and D), *SDHAF2* (succinate dehydrogenase assembly factor 2), and the more recently reported *TMEM127* (transmembrane protein 127) and *MAX* (myc-associated factor X;

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Translational Relevance

Succinate dehydrogenase (SDH) plays a key role in energy metabolism, which is deregulated upon loss of SDH function. Nearly 30% of pheochromocytomas and paragangliomas (PGL) are caused by germline mutations of which succinate dehydrogenase subunit B (SDHB) mutations have been associated with large-sized aggressive tumors and increased risk of malignancy. Thus, studies on energy metabolism in relation to endocrine activity are needed to improve diagnosis, localization, and treatment of these tumors. In such an attempt, we report a strong positive correlation between determinants of energy metabolism and catecholamine content in PGLs, suggesting a metabolic deviation in SDHB-related tumors that supports growth, contributing to larger sizes and implicating the need for targeting cellular energetics in therapy. Furthermore, we report increased succinate accumulation in these tumors, which could serve as a biomarker. Finally, genotype-specific differences in tumor metabolite contents reported in the study highlight the importance of metabolic imaging in tumor localization and patient follow-up.

ref. 2). In 17% of sporadic tumors, somatic mutations in RET, VHL, MAX, and more recently, HIF-2α and NF1 have been reported (3–5).

Based on transcriptional profiling studies, PGLs can be classified into 2 clusters: cluster 1 and cluster 2 (6, 7). Cluster 1 tumors (*VHL, SDHA/B/C/D/AF2)* are characterized by increased expression of genes involved in (pseudo) hypoxia, cell proliferation, angiogenesis, electron transport chain and the Krebs cycle, and abnormal function of oxidoreductases. Cluster 2 tumors (*RET, NF1*) show an increased expression of genes involved in protein synthesis, kinase signaling, endocytosis, and maintenance of a differentiated chomaffin cell catecholamine biosynthetic and secretory phenotype. Sporadic PGLs are distributed between the 2 major clusters based on their gene expression pattern and catecholamine phenotype (6).

SDH is an important component of the mitochondrial electron transport chain. In tumors with SDHx mutations, the ability of cells for oxidative phosphorylation is compromised (7–10). Also, it has been showed *in vitro* that accumulation of succinate in cells silenced for SDH causes inhibition of prolyl hydroxylase activity resulting in stabilization of hypoxia-inducible factor-1α (HIF-1α) and HIF-2α (11 and 12). HIF-1α and -2α then translocate to the nucleus where, together with aryl hydrocarbon receptor nuclear translocator, they form an active HIF complex that induces the expression of genes with hypoxia response elements that support tumor progression via different signaling pathways. Thus, in cluster 1 tumors, the pseudohypoxic drive is hypothesized to mediate an increase in aerobic glycolysis, also known as Warburg effect. This is supported by increased HIF-α protein level combined with lower SDH activity and increased glycolysis as indicated by lactate dehydrogenase activity (7).

The differences between cluster 1 and cluster 2 tumors are also characterized by differences in catecholamine biosynthetic and secretory profiles (13). PGLs with mutations in *RET* and *NF1* produce both epinephrine and norepinephrine and have low rate constants for catecholamine secretion, whereas *SDHx*- and *VHL*-related tumors mainly produce norepinephrine and have high rate constants for catecholamine secretion. Tumor catecholamine content is lower in cluster 1 tumors, compared to cluster 2 tumors. Also, it is well known that sequestration of catecholamines into chromaffin granules through vesicular monoamine transporters (VMAT) and re-uptake of catecholamines via norepinephrine transporter (NET) are active energy-dependent processes. Differences in catecholamine phenotypes may thus in part be explained by mutation-dependent changes in energy metabolism.

In this study, we therefore investigated relationships between genotype-specific differences in mitochondrial function and catecholamine content in PGL tumors. A total of 90 PGL tissues of various genotypes were included. Besides functional assays for respiratory complexes I to IV and citrate synthase, ^{1}H-nuclear magnetic resonance (NMR) spectroscopy was conducted to provide an overview of the intracellular metabolome with specific focus on catecholamines, ATP/ADP/AMP and intermediates of glycolysis and Krebs cycle.

Materials and Methods

Patients

Patients with histologically proven PGLs evaluated at the Department of Medicine, Division of Endocrinology of the Radboud University Nijmegen Medical Centre (RUNMC, Nijmegen, the Netherlands) and at *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), National Institute of Health (NIH) (Bethesda, MD) were considered for the study. Tumor tissue samples of consecutive patients from RUNMC who underwent surgical resection between 1988 and 2012 were included in the study. NIH patients underwent surgery between 2003 and 2010. Frozen primary tumor tissues from 64 patients at RUNMC and 26 patients at NICHD were included. The presence of germline mutations and large deletions in *SDHB/C/D*, *RET*, *VHL* and—since 2011—in *SDHA*, *SDHAF2*, *TMEM127*, and *MAX* were investigated using standard procedures. Data were collected under conditions of regular clinical care, with ethical committee approval obtained for the use of those data for scientific purposes at RUNMC. The study was approved by the Institutional Review Board of NICHD, and all patients gave written informed consent before testing. The details of the patients' clinical characteristics and genotype are listed in Table 1.

Tumor tissue processing

Tumor tissues resected from the patients described earlier were procured as early as possible, the dimensions of the

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Table 1. Patient characteristics

Genotype	*N* (patients)	Age, y (mean ± SD)	Gender (M/F)	*N* (tumors)	Tumor location (A/E/HN)	Tumor volume (cm^3)
Sporadic	35	47.5 ± 14.8	18/17	35	32/3/0	218.6 ± 452.5
SDHB	13	32 ± 11.9	10/3	15	1/14/0	273.1 ± 316.2
SDHD	8	44 ± 10.4	6/2	8	1/1/6	27.9 ± 47
SDHAF2	1	24	1/0	1	0/0/1	18.9
VHL	9	30.7 ± 13	6/3	9	7/2/0	61.5 ± 84.9
MEN-2	13	37.9 ± 12.8	6/7	15	15/0/0	93.4 ± 171.6
NF1	8	43.2 ± 17.4	5/3	8	8/0/0	107.6 ± 80.6
MAX	3	48 ± 14	2/1	3	3/0/0	63.2 ± 71.9

Abbreviations: A, adrenal; E, extraadrenal; F, female; HN, head and neck; M, male; N, no. of patients; y, years.

tumor were recorded by the pathologist and a small piece of the tumor tissue was weighed and snap frozen and stored in liquid nitrogen and later used for experimental purposes. For histologic confirmation, additional slices were stained with hematoxylin and eosin and re-evaluated by an independent pathologist (B. Kusters).

Respiratory chain enzyme assays

Frozen tumor specimens (~40 mg) were homogenized on melting ice in sucrose–EDTA–phosphate buffer [0.25 M sucrose, 2 mmol/L EDTA, 10 mmol/L K_2PO_4, pH 7.4, 8% (w/v)] using a hand-held glass/glass homogenizer. Homogenates were subsequently centrifuged at 600 × *g* at 4°C for 10 minutes and supernatants used for the determination of the activities of respiratory chain enzyme complexes I to IV and the mitochondrial matrix enzyme, citrate synthase. These assays measured the formation of a spectrophotometrically detectable end-product at regular time intervals and were conducted on Konelab 20XT clinical chemistry analyzer (Thermo Scientific) as described elsewhere (14). The protein concentrations in the supernatants were also measured in parallel using pyrogallol red–molybdate complex method as described earlier (15). The enzyme activities were normalized to mg protein. Five samples were excluded from analysis (2 Sporadic; 1 each SDHB, VHL, and NF1) as the tumor tissue homogenate contained blood which could affect determinations and interpretations of respiratory chain enzyme activities and protein concentrations.

^{1}H-NMR spectroscopy

^{1}H-NMR spectroscopy was conducted in frozen tumor tissues to determine concentrations of intermediates of energy metabolism (Krebs cycle and glycolysis), catecholamines, and their metabolites. One sporadic, 2 SDHB, 1 VHL, and 2 MAX tumors were excluded from the experiment as the amount of starting material was low. The tumor tissues were homogenized on ice in 10% (w/v) of distilled water using a hand-held Teflon/glass homogenizer. The samples were then centrifuged at 16,000 × *g* for 10 minutes at 4°C and the supernatants were subjected to ultrafiltration with Vivaspin Turbo 15, 10 kDa filters (Sartorius). The ultrafiltrates were diluted with water to 700 µl pH was adjusted to 2.5 and 20 µL of 20.2 mmol/L sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate in D_2O was added to the samples. The samples were then placed in 5 mm NMR tubes and ^{1}H-NMR spectra were obtained using a Bruker 500 MHz spectrometer (pulse angle 90°, 7 microseconds pulses with a delay time of 4 seconds, number of scans 256). The water resonance was suppressed by gated irradiation centered on water frequency (16, 17).

Concentrations of succinate in the tumor tissues were estimated by integrating the area under the peak at 2.66 ppm. Differences in peak heights were clearly observed for tumors with high, absent, and low levels of succinate (Supplementary Fig. S1). Tumor tissue ATP/ADP/AMP content was estimated by integrating the area under peaks in the region 6.18 to 6.21 ppm. Because each of the 3 compounds differs by the presence of one phosphate group, spectral peaks for these metabolites cannot be distinguished using ^{1}H-NMR spectroscopy. Epinephrine content was estimated by integrating area under the peaks for the triplet at 2.73 ppm and the total catecholamine content was estimated by integrating the area under the peaks for multiplets in the region 6.86 to 6.98 ppm (Supplementary Fig. S1). Furthermore, norepinephrine content was estimated by calculating the difference between total catecholamine and epinephrine contents. Other amines, which could contribute to peaks in the region 6.86 to 6.98 ppm are dopamine and 3-methoxytyramine. However, the concentration of these compounds as measured by high-performance liquid chromatography (HPLC) is in the nanomolar range (18), much lower than the detection limit of ^{1}H-NMR spectroscopy, which is in micromolar range. The catecholamine content of the tumor tissue as estimated by ^{1}H-NMR spectroscopy was validated in a small subset of 22 samples using HPLC. For this purpose, the frozen tumor tissues (~5 mg) were weighed accurately, transferred to a processing tube and 5 volumes of 0.4 M perchloric acid containing 0.5 mmol/L EDTA is added and homogenized on ice using a homogenizer (Polytron). The tubes are then spun for 15 minutes at 3,000 rpm in a refrigerated centrifuge to separate the precipitated proteins and cell debris. The perchloric acid extract is separated from the pellet, frozen on dry ice, and stored at −80°C until assayed for catecholamines. Furthermore, the

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perchloric acid extract is prepared using alumina extraction method as described previously (19). A significant correlation and linear relationship was observed between the 2 methods (Supplementary Fig. S2).

Statistical methods

Statistical analyses were conducted using SPSS (SPSS Inc., v.18) and GraphPad Prism 6 software (GraphPad). The data were analyzed using independent samples Kruskal–Wallis test and Dunn post test was used to compare the different genotypes and adjusted P values were reported. Correlation between respiratory chain enzyme activities and tumor tissue catecholamine content was examined using Spearman correlation test. Statistical significance was accepted at P value < 0.05. Furthermore, estimation of catecholamine content by ^{1}H-NMR spectroscopy and HPLC and comparison of respiratory chain enzyme activities with total catecholamine content was carried out using Passing–Bablok regression analysis (20).

Results

Respiratory chain enzyme activities

The activity of the respiratory chain complex II was deficient in all SDHx tumors as expected (Fig. 1B; $P < 0.0001$). The activity of the complexes I, III, and IV was significantly higher in SDHx tumors than in VHL, RET, NF1, and sporadic tumors (Fig. 1A, C, and D; $P < 0.05$). This was even more clearly so for citrate synthase activity (Fig. 1E; $P < 0.0001$). The VHL tumors showed a lower activity for complex I when compared to sporadic tumors (Fig. 1A and Table 2; $P < 0.05$) and complex III when compared to sporadic (Fig. 1C and Table 2, $p < 0.01$) and cluster II tumors (Fig. 1C and Table 2; $P < 0.05$). In contrast, the MAX tumor group had a tendency of higher activity for the complexes II, III, and IV when compared to the other genotypes (Fig. 1B–D). Furthermore, for the various SDH mutations no mutation specific differences could observed in the activities of respiratory chain enzyme complexes I to IV. Although a low complex II activity was observed in SDHB-related tumors when compared to SDHD-related ones, the sensitivity of the assay at such low enzyme activity levels precludes such an analysis and interpretation of the results (Supplementary Fig. S3).

Detection of energy metabolism intermediates and tumor tissue catecholamines using ^{1}H-NMR spectroscopy

The NMR spectra of the tumor homogenates showed very high succinate ($P < 0.001$) levels in all SDHx cases as

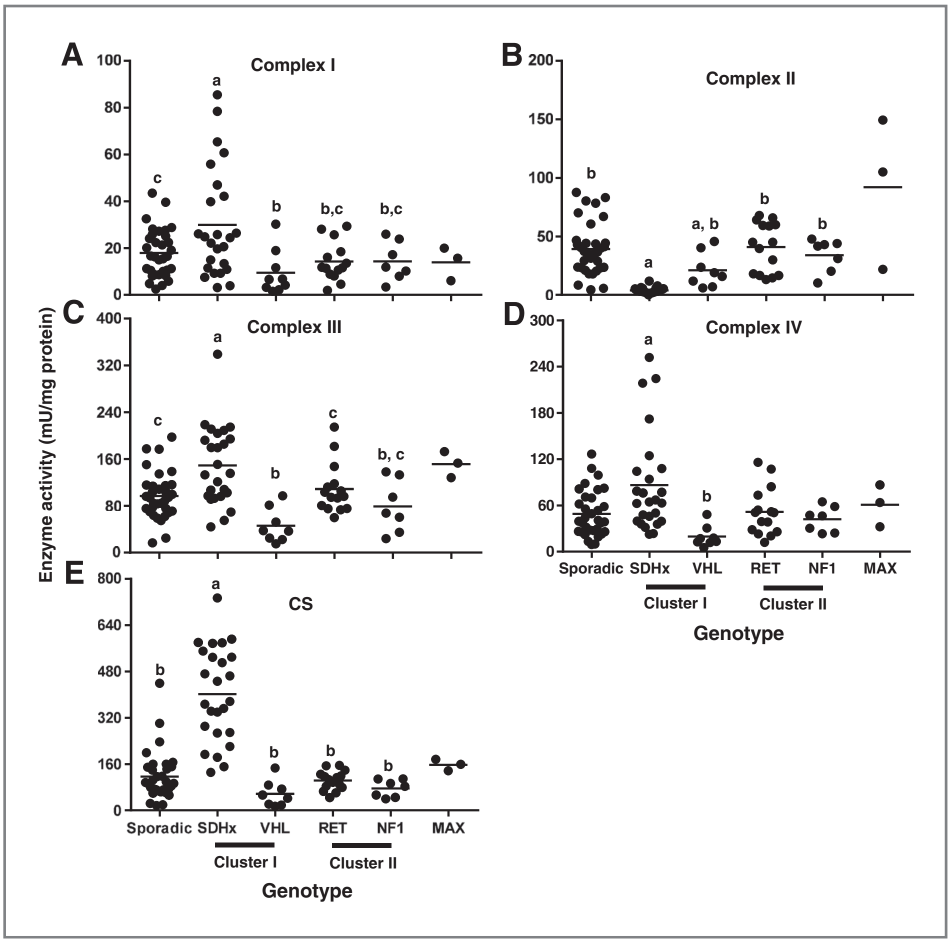

Figure 1. Respiratory chain enzyme activity in PGL tumor tissues of different genotypes. A–E, dot plots depicting the respiratory chain enzyme activities of respiratory chain enzyme complexes I to IV and citrate synthase (CS) across different genotypes in mU normalized to mg protein concentrations. Horizontal line represents the mean. Datasets having different alphabets above them are significantly different ($P < 0.05$).

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Table 2. Comparison of respiratory chain complex activities between different genotypes

Genotype	SDHx vs.		VHL vs.	
	Sporadic	MEN-2 and NF1	Sporadic	MEN-2 and NF1
Complex I	0.3164	**0.0278**	**0.0478**	0.4221
Complex II	**<0.0001**	**<0.0001**	0.1150	0.1703
Complex III	**0.0050**	**0.0165**	**0.0082**	**0.0137**
Complex IV	**0.0397**	**0.0439**	0.0600	0.0733

NOTE: Listed in the table are *P* values (adjusted) for the various comparisons. Highlighted in bold letters are comparisons that attained statistical significance.

expected (Fig. 2A). Mutation specific differences in the levels of succinate could not be observed for the tumors with various SDH mutations (Supplementary Fig. S3). Succinate was not NMR detectable or very low in all other tumor samples except for 1 tumor in the sporadic group. Citrate was present in high concentration in 4 sporadic tumors. Low concentrations of pyruvate, without genotype-specific differences, were observed in all PGL tissues (data not shown).

The differences in high-energy phosphate content between the tumors were striking. Proton NMR spectroscopy cannot discriminate between ATP, ADP, and AMP and therefore Fig. 2B shows the sum of the 3 high-energy phosphates. The concentration of ATP/ADP/AMP was consistently very low ($P < 0.0001$) in all SDHx tumors. A very low content also occurred in the other tumor groups but the ATP/ADP/AMP concentration was rather variable in these groups. RET tumors had high ATP/ADP/AMP when compared to sporadic ($P < 0.01$) and SDHx tumors ($P < 0.0001$).

Epinephrine was proton NMR—undetectable in all SDHx and VHL tumors. The RET tumors showed high epinephrine concentrations when compared to sporadic ($P < 0.05$),

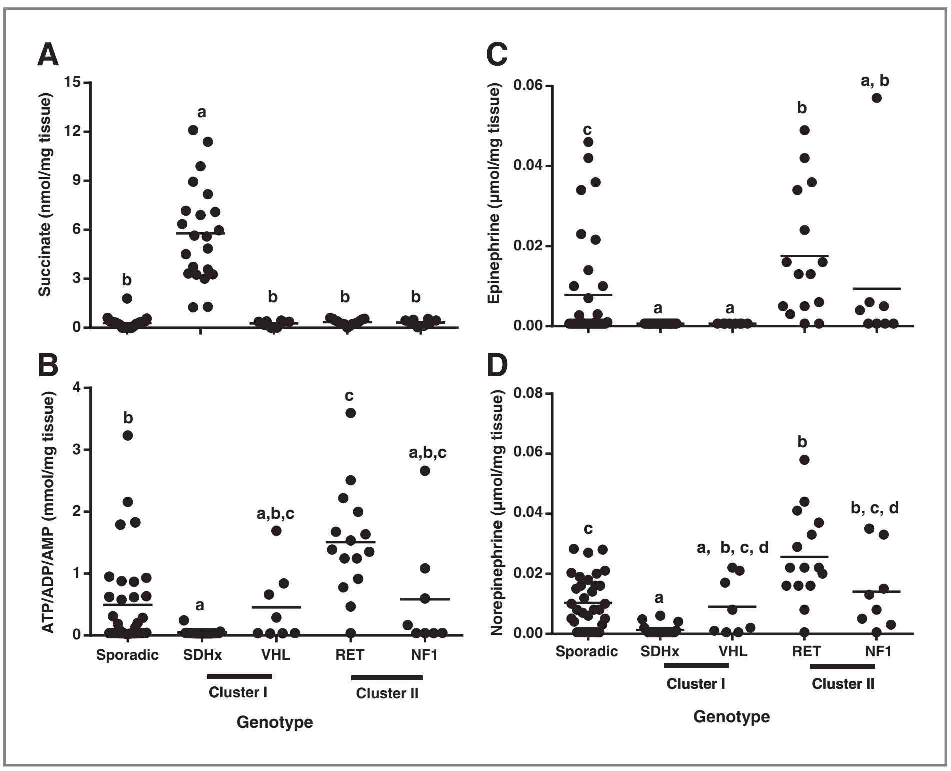

Figure 2. Accumulation of intermediates of energy metabolism and catecholamine metabolism in PGL tumor tissues of different genotypes as determined by ^{1}H-NMR spectroscopy. A, dot plot depicting the tumor tissue succinate concentrations expressed as nmol per mg tumor tissue across different genotypes. B, dot plot depicting the tumor tissue ATP/ADP/AMP concentrations expressed as mmol per mg tumor tissue across different genotypes. C, dot plot representing the tumor tissue epinephrine concentrations expressed as µmol per mg tumor tissue across different genotypes. D, dot plot representing the tumor tissue norepinephrine concentrations expressed as µmol per mg tumor tissue across different genotypes. Horizontal line represents the mean. Datasets having different alphabets above them are significantly different ($P < 0.05$). In 13 tumor samples succinate and ATP/ADP/AMP and in 33 tumor samples total catecholamine peaks were below detection limit. They have been represented as half the lowest detectable value.

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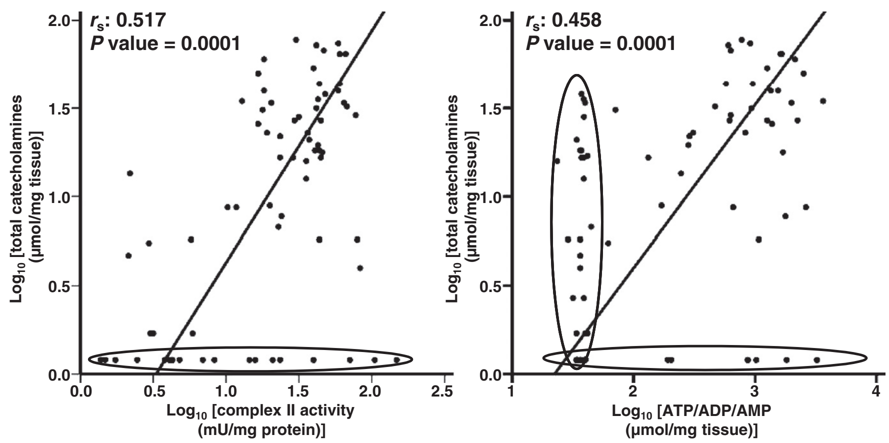

Figure 3. Relationship between tumor tissue catecholamine content and activity of respiratory chain enzyme complex II and tumor ATP/ADP/AMP content. Passing–Bablok regression plots for activities respiratory chain enzyme complex II (mU per mg protein) vs. total catecholamine content (nmol per mg tissue) in PGL tumor tissues represented in $\log_{10}$ scale. Points for samples below detection limit of ^{1}H-NMR spectroscopy are encircled in black.

SDHx ($P < 0.0001$), and VHL ($P < 0.001$) tumors (Fig. 3C). In SDHx tumors, norepinephrine was also very low when compared to sporadic ($P < 0.01$), RET ($P < 0.0001$), and NF1 tumors ($P < 0.05$), whereas 50% of the VHL tumors produced significant amounts of norepinephrine (Fig. 3D). Total catecholamine peaks were below detection limit in 33 tumor samples. Seven of these samples were subjected to HPLC, which detected the presence of catecholamines in these tissues.

Correlation of energy metabolism with tumor tissue catecholamine content

Positive correlations ($P < 0.001$) were observed between activities of respiratory chain complex II and concentrations of epinephrine, norepinephrine, and total catecholamine content in tumor tissues. ATP/ADP/AMP content of PGL tumors also showed a positive correlation with tumor tissue epinephrine, norepinephrine, and total catecholamine levels (Table 3). Parasympathetic PGLs were excluded from this analysis as they do not produce catecholamines.

Furthermore, Passing–Bablok regression statistics for comparison between complex II activity, tumor ATP/ADP/AMP content, and total catecholamine content showed a linear relationship (Fig. 4).

Discussion

This study establishes differences in mitochondrial energy pathways in metabolic processes in PGLs and provides novel insight into how deregulation of energy metabolism might impact catecholamine phenotypic features of cluster 1 and cluster 2 tumors. Furthermore, the study also provides *ex vivo* evidence for the accumulation of succinate in SDH-related tumors and successfully uses ^{1}H-NMR spectroscopy for detection of catecholamines in PGL tumor tissues.

In accordance with previous reports (7–10), we observed that the activity of SDH or respiratory chain enzyme complex II is low in SDH-related tumors. Interestingly, reduction of complex II activity in SDH-related tumors was associated with increased activities of other respiratory chain complexes I, III, and IV and citrate synthase. The

Table 3. Correlation of respiratory chain enzyme activity with tumor tissue catecholamine content

	Catecholamines		CI (mU/mg protein)	CII (mU/mg protein)	CIII (mU/mg protein)	CIV (mU/mg protein)	ATP/ADP/AMP (mmol/mg tissue)
Spearman ρ	E (nmol/mg tissue)	Correlation coefficient	0.226	**0.423**	0.182	0.006	**0.366**
		Sig. (2-tailed)	0.046	**0.0001**	0.097	0.955	**0.0001**
		N	78	**78**	78	78	**82**
	NE (nmol/mg tissue)	Correlation coefficient	0.160	**0.479**	0.078	0.066	**0.390**
		Sig. (two-tailed)	0.163	**0.0001**	0.495	0.569	**0.0001**
		N	78	**78**	78	78	**82**
	Total catecholamines (nmol/mg tissue)	Correlation coefficient	0.208	**0.517**	0.148	0.033	**0.458**
		Sig. (two-tailed)	0.067	**0.0001**	0.196	0.774	**0.0001**
		N	78	**78**	78	78	**82**

NOTE: Highlighted in bold letters are comparisons that attained statistical significance.
Abbreviations: CI, II, III and IV, respiratory chain enzyme complexes I to IV; E, epinephrine.

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increased citrate synthase activity also indicates an increase in the mitochondrial content. This observation is in agreement with report by Douwes and colleagues (21) in which elevated numbers of tightly packed mitochondria were observed in SDHD linked head and neck PGLs. A similar increase in activities of complexes I and III and citrate synthase activities in SDH-related tumors when compared to VHL-related ones was observed by Fliedner and colleagues (22). All these factors suggest a compensatory response to the lack of SDH activity and associated activity of complex II. However, as currently shown, the apparently increased activity of complexes I, III, and IV and citrate synthase in the tumors does not lead to full restoration of ATP/ADP/AMP. This contrasts with the report of Favier and colleagues (7) in which no differences for complex III activity were observed among NF1/RET, SDH, and VHL-related PGLs and that of Rapizzi and colleagues (10), where no differences in citrate synthase activity were observed across different genotypes. Our findings of higher complex IV activity in SDH-related tumors than NF1- and RET-related ones contradicts with the findings by Favier and colleagues (7) who observed a decreased complex IV activity in SDHx and VHL-related tumors. However, our observations are in agreement with the increased expression of complex IV protein reported in 4 of 5 SDH-related tumors by Rapizzi and colleagues (10). Nevertheless, this study also indicated a high degree of variability in complex IV activities in these tumors.

Previous studies by Lopez-Jimenez and colleagues (23) and Favier and colleagues (7) have showed that there is a preferential activation of HIF-1α target genes such as glycolytic pathway enzymes in VHL tumors whereas activation of HIF-2α is observed in both VHL and SDHx tumors. It is also thought that activation of hypoxic response may be directly involved in decreased mitochondrial respiration. Interestingly, in this study we observed an increase in complexes I, III, and IV and citrate synthase in SDHx tumors whereas there was a trend of overall downregulation of respiratory chain activities in VHL tumors. Also, we observed that in contrast to VHL tumors, accumulation of ATP/ADP/AMP in SDHx tumors was undetectable (except in one sample). This indirectly supports the observations by Favier and colleagues (7) that there is an activation of glycolysis as evidenced by increased lactate dehydrogenase activity in VHL tumors and not SDHx tumors. However, increased expressions of GLUT-1, GLUT-3, and Hexokinase II mRNAs observed in SDH tumors (7) can explain the high sensitivity of [^{18}F]-FDG PET to SDH tumors (24–26).

In *in vitro* experiments with cells silenced for SDH, it was observed that the accumulation of succinate leads to stabilization of HIF-1α (11). Later, Pollard and colleagues (27) described succinate accumulation in tumor tissues with germline SDH mutation, however, it was described in a single patient with pathogenic SDH mutation. In this study, we for the first time provide strong *in vivo* evidence to support the hypothesis that there is an accumulation of succinate in SDH-related PGL tumor tissues. This supports the concept that stabilization of HIF-α in SDH-related tumors reflects the inhibitory effects of succinate on prolyl hydroxylase. High succinate accumulation observed in tumors with SDHx mutations also makes this a reliable parameter to indicate mutations in SDH subunits or assembly factors.

Genotype-specific differences in catecholamine production by PGLs are well known. In this study, our observations that tumors with mutations in VHL and SDHx mainly produce noradrenaline and those with mutations in RET and NF1 produce both adrenaline and noradrenaline support the previous findings by Eisenhofer and colleagues (13). This difference in the catecholamine phenotype has been attributed to the lack of the enzyme phenylethanolamine *N*-methyltransferase in VHL and SDHx tumors (28). In line with the previous studies (13), we also observed that the tumor tissue total catecholamine content was lower in SDHx tumors when compared to RET and sporadic PGLs. These differences could be possibly attributed to the increased tyrosine hydroxylase activity in RET tumors (28) however, tyrosine hydroxylase activity or expression has not been investigated in SDHx tumors in comparison with other genotypes albeit a lack of the expression of this enzyme has been observed in biochemically silent SDHB tumors (29).

In this study, we established that tumor tissue concentrations of ATP/ADP/AMP, as determined by low peak heights at relevant resonance positions in ^{1}H-NMR spectra, are lower in SDH-related tumors than in other tumors. Furthermore, our findings of positive relationships between respiratory enzyme complex II function, tumor ATP/ADP/AMP content, and tumor catecholamine contents suggest the possibility that differences in energy metabolism might also contribute to the lower tumor tissue catecholamine contents in cluster 1 than in cluster 2 tumors. To this end, it is well known that the sequestration of catecholamines into secretory vesicles and re-uptake of catecholamines into chromaffin cells are active energy dependent processes. Sequestration of catecholamines is facilitated by VMATs. The H^{+} gradient necessary to maintain the activity of VMATs is generated by ATP-dependent vesicular membrane proton pump (30). Chromaffin granules also contain strikingly high concentrations of ATP due to the activity of vesicular nucleotide transporter (31). This contributes to the stability and ability of chromaffin granules to maintain stores of catecholamines (32, 33). Furthermore, NET responsible for the sodium chloride (Na^{+}/Cl^{-})–dependent reuptake of extracellular norepinephrine and dopamine is also indirectly dependent on cellular store of ATP. NET functions by coupling the transport of norepinephrine and dopamine with the influx of sodium and chloride (Na^{+}/Cl^{-}). The ion gradients of Na^{+} and Cl^{-} generated by the Na^{+}/K^{+}-ATPase make this reuptake energetically favorable (34, 35). Clearly therefore, energy metabolism has an important role in maintaining the stability of chromaffin granules and thus catecholamine storage. It thereby seems possible that genotype-specific differences in the energy metabolism, along with associated differences in expression of various genes encoding components of secretory pathway and exocytotic machinery (36), could in conjunction contribute to genotype-specific differences in tumor catecholamine phenotypic features.

In this study we included 35 sporadic tumors, 60% of which were not tested for SDHA and SDHAF-2. Two of the 3 sporadic tissues which had low respiratory chain complex II activities comparable to SDHx tumors also belong to the group that were not tested for SDHA and SDHAF-2. Thus, mutations in SDHA and SDHAF-2 cannot be ruled out in these tumors. Also, the low activity may indicate that these tumors may have an as yet unidentified intronic or promotor mutations in SDH subunit genes or unidentified mutations in assembly factors genes.

We used ^{1}H-NMR spectroscopy to determine the tumor tissue metabolite concentrations as it provides a holistic view on the tumor metabolome. This technique can very well identify various metabolites and quantify differences in the metabolite concentrations among different samples, but it is limited by its sensitivity. It can quantify metabolites only in micromolar range because of which many intermediates of energy and catecholamine metabolism could not be determined in this study. This is clearly visible in the Passing–Bablok regression analysis of total catecholamines versus complex II activity and ATP/ADP/AMP levels, where reduced sensitivity of NMR spectroscopy separates out a group of samples which if analyzed with a more sensitive method could have reflected the linear relationship better. Nevertheless, the study was successful in identifying the relationship between catecholamine content of PGLs and energy metabolism.

Catecholamine contents are particularly low in tumors due to SDHB mutations and it has been suggested that this along with diversion of energy from maintaining catecholamine phenotypic features to growth might contribute to the larger sizes and more aggressive features of these tumors (18). This study, establishing relationships between tumor energetics and catecholamine phenotypic features, provides new insight into how such diversions of energy might occur with implications for novel therapeutic strategies targeting energy pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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