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Alterations of respiratory chain complexes in sporadic pheochromocytoma

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1. ABSTRACT

Succinate dehydrogenase (SDH) has been associated with carcinogenesis in hereditary pheochromocytoma (PC) and paraganglioma. We investigated if a similar association applies to sporadic pheochromocytoma. No genetic alteration was found in the *SDHB, SDHC* or *SDHD* genes of sporadic PC. However, in eight of nine sporadic PCs the SDH activity was, on average, reduced by 40%; moreover, the activities of the other oxidative phosphorylation (OXPHOS) complexes and citrate synthase were significantly lower compared to normal kidney tissue. Furthermore, immunohistochemical staining revealed a significant down-regulation of respiratory chain complexes. Since no pathogenic mutations were detected in the von Hippel-Lindau (VHL) gene, we can rule out that VHL deficiency is causing the general reduction of OXPHOS enzymes observed in the PCs investigated. In contrast to the single enzyme defects found in a subset hereditary PCs, a more generalized reduction of mitochondrial respiration seems to be present in most sporadic PCs. Strikingly, one of the nine PCs showed specific loss of complex I and a compensatory up-regulation of complexes II-V, which is a phenotype usually characteristic of oncocytic tumors.

2. INTRODUCTION

Pheochromocytomas (PCs), paragangliomas (PGLs), neuroblastomas (NBs), ganglioneuroblastomas (GNBs) and ganglioneuromas (GNs) derive from neural-crest cells. Their common origin is underlined by reports of composite PGL-GN, PGL-NB, PGL-GNB and PC-NB (1-3). PCs generally arise from the adrenal medulla, and PGLs from either the sympathetic nervous system or parasympathetic ganglia (4). In contrast to neuroblastic tumors, PCs and PGLs contain mature chromaffin cells. They are usually benign, catecholamine- secreting tumors. Plasma catecholamines, serum chromogranin, urinary free catecholamines, and plasma metanephrins are used in the diagnosis of PC.

PCs are associated with several familial syndromes, including multiple endocrine neoplasia (MEN) type 2A, von Recklinghausen's neurofibromatosis and other endocrinopathies (5). Mutations in the von Hippel Lindau (VHL) gene predispose to a variety of malignant and benign neoplasms, including clear cell renal carcinoma and PC (6-8). Hereditary PGL and PC frequently exhibit germline mutations in the succinate dehydrogenase (SDH) subunits SDHB, SDHC and SDHD, indicating that these

SDH subunits act as tumor suppressors in neuroendocrine tissues (9). Carriers of SDH mutations usually develop a tumor earlier in life and have a more severe phenotype than do patients with no germline mutations in these genes. SDHB mutations are often identified in patients with no family history whereas patients with SDHD mutations often have a paternal family history of PC (10). The SDHA subunit is mutated in patients with Leigh syndrome and late-onset optic atrophy, but has not as yet been identified as a factor in hereditary cancer.

However, the majority (~70%) of PCs do not have a detectable mutation in any of the known susceptibility genes and are considered as sporadic tumors (11). Several reports in the past five years have established that approximately 12-16% of apparently sporadic PCs or PGLs carry a germline mutation in one of the well-known susceptibility genes (11-14). Recent biochemical evidence suggests that PC-susceptibility mutations (RET, VHL, SDHB, SDHC, SDHD, NF1) converge into a common signaling defect that abrogates the prolyl hydroxylase Egln3/PHD3-mediated apoptotic response of sympathetic neurons to limiting amounts of nerve growth factor (NGF) during development (15). This model predicts that cells that escape this physiological developmental death would later give rise to PC and PGL.

The use of anaerobic fermentation of glucose as an energy source through glycolysis is a feature common to many solid tumors, in turn leading to a lesser dependence on oxidative phosphorylation (OXPHOS), which is called the Warburg effect (16, 17). The down-regulation of OXPHOS in tumor cells seems to be achieved by different mechanisms. First, profound hypoxia can be the cause of compensatory up-regulation of glycolysis in most tumors. Second, it is becoming more and more evident that the loss of tumor suppressor genes such as VHL and p53 or activation of oncogenes results in down-regulation of OXPHOS (18, 19). Finally, as indicated above, direct inactivation of components of OXPHOS has been detected in a minority of tumors (PC, renal oncocytomas, thyroid oncocytomas) (20-23).

In light of the known alterations of SDH in hereditary PC, the aim of the present study was to elucidate specific alterations of aerobic energy metabolism in sporadic cases of PC.

3. MATERIAL AND METHODS

3.1 Ethics Statement

The study was performed according to the Austrian Gene Technology Act. Experiments were performed in accordance with the Helsinki Declaration of 1975 (revised 1983) and the guidelines of the Salzburg State Ethics Research Committee, being no clinical drug trial or epidemiological investigation. All patients gave informed written consent concerning the surgical removal and therapy of the tumors. Furthermore, the study did not extend to examination of individual case records. The anonymity of the tumor samples has been ensured.

3.2. Samples

Frozen PC tissues (n=9) were obtained from the University Hospital Salzburg, Austria, and the Biobank, Graz, Austria. Fourteen unaffected kidney tissues served as references for enzymatic measurements. In addition, for immunohistochemical studies, formalin-fixed, paraffin-embedded PCs (n = 10) and unaffected adrenal tissues were used.

3.3. Enzyme measurements

All tissues were frozen and stored in liquid nitrogen within 30 min after surgery. Tumor cell content and cellular composition of samples were evaluated using hematoxylin-eosin-stained frozen sections. In tissue samples with a tumor cell content >90% the enzymatic activities of OXPHOS complexes and citrate synthase were analyzed as described previously (24).

3.4. Sequencing of VHL

DNA was isolated using a NucleoSpin® Tissue Kit (Macherey-Nagel). 100 ng genomic DNA was used for the PCR amplification. PCR products were incubated with Exo SAP IT 500 (USB Corporation), and the CEQ DTCS Quickstart Kit (Beckman Coulter) was used for the sequencing reaction using the PCR primers listed in Table 1, followed by separation with a CEQ 2000 DNA Analysis System (Beckman Coulter).

3.5. Quantitative PCR for the detection of LOH in SDHB, SDHC or SDHD

Quanta Biosciences B-R SYBR® Green SuperMix was used for the quantitative PCR to detect loss of heterozygosity (LOH) in SDHB, SDHC or SDHD. The following PCR protocol was used: 1 cycle: 95°C 3 min; 40 cycles: 95°C 2 sec, 65°C 20 sec, 72°C 10 sec; 1 cycle: 95°C 1 min; 1 cycle: 55°C 1 min. Primers are listed in Table 1.

3.6. Sequencing of mitochondrial complex I and tRNA genes

The primers used for amplification and sequencing are listed in Table 1.

3.7. Western blot analysis

Western blot analysis of 600*g* homogenates was performed as described previously (24).

3.8. Immunohistochemical staining and analysis

Immunohistochemical staining was performed as described previously (22) using the following antibodies: anti-complex I subunit NDUFS4, anti-complex II subunit 70kDa Fp, anti-complex III subunit core protein 2, mouse monoclonal anti-complex IV subunit I, mouse monoclonal anti-complex V subunit alpha, and mouse monoclonal anti-porin 31HL.

4. RESULTS

Loss of heterozygosity (LOH) in combination with germline SDH mutations are found in a subset of hereditary PC but not sporadic PC. To rule out that the PC tissues used in this study do not also have LOH in *SDHB, SDHC*

Table 1. Primers used for amplification, sequencing and RT-PCR of mitochondrial DNA and nuclear genes

	position	forward primer sequence	reverse primer sequence	length
mtDNA	548-964	GGGAGGGGGTGATCTAAAAC	CCAACCAAACCCCAAAGAC	416
mtDNA	1487-1952	GCCCGTCACCCTCCTCAAG	ACGGGTGTGCTCTTTTAGCTG	465
mtDNA	3079-3505	TAGATGTGGCGGGTTTTAGG	GGAGTAATCCAGGTCGGTT	426
mtDNA	3439-3894	GCTACTACAACCCTTCGCTGAC	GTTCGGTTGGTCTCTGCTAGTG	456
mtDNA	3734-4235	CTGCGAGCAGTAGCCCAAAC	TGCTAGGGTGAGTGGTAGGAAG	501
mtDNA	4183-4552	TTCCTACCACTCACCCTAGCA	AAAAATCAGTGCGAGCTTAGC	369
mtDNA	4506-5004	CATCTTTGCAGGCACACTCATC	GATTTTGCGTAGCTGGGTTTGG	499
mtDNA	4956-5484	CATAGCAGGCAGTTGAGGTGG	AGGTAGGAGTAGCGTGGTAAGG	529
mtDNA	5429-5929	CAAAACCCACCCCATTCCTCC	AATAGTCAACGGTCGGCGAAC	500
mtDNA	6955-7492	GGTGGCCTGACTGGCATTG	GTTGGCTTGAAACCAGCTTTGG	537
mtDNA	7401-7682	ACCCTACCACACATTCG	GGAAAATGATTATGAGGGCG	281
mtDNA	8012-8561	AGTACTCCCGATTGAAGCCCC	GGGCAATGAATGAAGCGAACAG	550
mtDNA	9916-10367	AGCCGCCGCCTGATACTG	CCAGACTTAGGGCTAGGATGATG	451
mtDNA	10279-10634	CCCTACCATGAGCCCTACAAAC	TAAGAGGGAGTGGGTGTTGAGG	356
mtDNA	10535-10926	AGTCTAGGCCATATGTGTTGGAG	AGGTTGGGGAACAGCTAAATAGG	391
mtDNA	10832-11315	ATCAACACAACCACCCACAGC	GTTCTTGGGCAGTGAGAGTGAG	484
mtDNA	11188-11720	TGAACGCAGGCACATACTTCC	TCCGTGGGCGATTATGAGAATG	533
mtDNA	11654-12195	ACAGCCATTCTCATCCAAACCC	GGTCGTAAGCCTCTGTTGTCAG	542
mtDNA	12009-12462	GCTCACTCACCCACCACAT	GCTCACTCACCCACCACAT	454
mtDNA	12358-12848	ACCACCCTAACCCTGACTTCC	GCTTGAATGGCTGCTGTGTTG	491
mtDNA	12806-13311	GATGATACGCCCGAGCAGATG	TGCTAGGTGTGGTTGGTTGATG	506
mtDNA	13250-13786	TCCACTTCAAGTCAACTAGGAC	GGGGATTGTTGTTTGGAAGGG	537
mtDNA	13708-14070	GCAGCCGGAAGCCTATTCG	TGAGGTGATGATGGAGGTGGAG	363
mtDNA	13931-14373	ATCACACACCGCACAATCCC	ATTGGTGCTGTGGGTGAAAGAG	442
mtDNA	14262-14707	TCCTCCCGAATCAACCCTGAC	TCATTGGTCGTGGTTGTAGTCC	446
mtDNA	14548-14992	AATAACACACCCGACCACAC	AAGGTAGCGGATGATTCAGC	445
mtDNA	15588-16187	CTCCGATCCGTCCCTAACAAAC	GGTTTTGATGTGGATTGGGT	598
mtDNA	52-323	GGGTCCTCAGTGGATGTAGGCT	GCCTTGCCCTATGCTTCCTC	272
mtDNA	9142-9518	GAATGCCTGCCTTTTCTAAGAAGA	GCCATCGGATGATCTCAGATTTT	377
SDHB	26201-26522	CGAGTAGTCAGTGTCCAAGAAATGG	AATGGCTTGCATCAGCTTATGTTC	317
SDHC	13920-14255	CCTGGCTTGGTATTGCAAAATATTG	AAGGGTTCACCTCATCTACATAGCA	336
SDHD	1870-2214	TGTACACTGCCTGTCAGTTTGGG	TAGGGCATTTCAATCAACTTCTCCC	345
VHL	86-609	GTTCCATCCTCTACCGAGCG	GGCTTCAGACCGTGCTATCG	523
VHL	4845-5119	TGTGGCTCTTTAACAACCTTTGC	GATTGGATAACGTGCCTGACATC	274
VHL	8101-8415	GCAAAGCCTCTTGTTCGTTCC	AAGGAAGGAACCAGTCCTGTATC	314

Position: Position of the PCR fragments. Length: Length of the PCR products. The following reference sequences have been used for the positions: mtDNA (J01415), SDHB, SDHC (NC_000001.10), SDHD (NC_000011.9), VHL (NM_000551.2)

and *SDHD* we analyzed DNA isolated from the tumor tissue by quantitative PCR. None of the tumor tissues had LOH in *SDHB, SDHC* and *SDHD*.

Although there was no genetic evidence for specific alteration of SDH, we analyzed the enzymatic activity of SDH in nine tissues of sporadic PC (Table 2). The SDH activity was significantly down-regulated in 8 of 9 PCs ($P<0.0001$) compared to control kidney tissues. Western blot analysis also revealed a reduction of SDH in eight of nine cases (Figure 1). To test if sporadic PCs are characterized by an isolated SDH defect, we determined the enzymatic activity of the other OXPHOS complexes. A significant down-regulation of the total activity of complex I was found in all cases. Complex III and complex IV activity was also significantly lower in eight of the nine PCs compared to normal adrenal gland and kidney tissue (Table 2). Complex V was less affected. In addition, a significant reduction of citrate synthase activity was observed in eight of the nine PCs compared to normal cortical kidney tissues (Table 2).

Case 8 showed down-regulation of complex I activity with concomitant enormous up-regulation of citrate synthase and the other OXPHOS complex activities (Table 2; Figure 1). Because of the unique biochemical signature we decided to exclude this case from further statistical analysis. The biochemical features of case 8 seem to resemble those of oncocytic tumors (21, 22). Accordingly, case 8 showed a 21-fold increase in citrate synthase activity, a 14-fold increase in complex II activity, a 2-fold increase in complex III activity, a 17-fold increase in complex IV activity, and a 6.5-fold increase in complex V activity compared to the other PCs. In addition, immunoblot analysis showed a severe reduction of the complex I subunit NDUFS4 in case 8, whereas the SDHA subunit was massively up-regulated (Figure 1). Mitochondrial DNA-encoded complex I and tRNA genes were sequenced in case 8, but no pathogenic mutations were found. However, we cannot rule out that a mutation in one of the numerous nuclear complex I genes causes the observed deficiency in case 8.

To substantiate our biochemical analysis we also analyzed further sporadic cases of PC by immunohistochemistry (Figure 2). In agreement with the enzymatic analysis, immunohistochemical staining also revealed a significant reduction of OXPHOS complexes I to IV in comparison to adjacent adrenal gland tissues ($P=0.0005$). This finding was further underlined by Western blot analysis of complex I and complex II subunits (Figure 1). No significant alterations were found in PC regarding complex V through immunohistochemical staining compared to adjacent adrenal gland (Figure 2; Table 3). This is also in accordance with the observed enzymatic activity of complex V in PC, which is also less severely affected than the activities of the other complexes.

Table 2. Enzymatic activity of OXPHOS enzymes and citrate synthase in pheochromocytomas

mUnits / mg protein	Kidney[1] (n=14)	Adrenal gland (n=4)	Tumor[2] (n=8)	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Citrate synthase	110 ± 7	243 ± 61	65 ± 0	45	42	151	58	47	49	101	1375	27
Complex I	43 ± 2	14 ± 3	7 ± 2	8	3	17	6	7	3	12	3	2
Complex II	128 ± 12	83 ± 10	13 ± 4	14	3	33	12	14	9	21	201	1
Complex III	161 ± 10	173 ± 11	117 ± 16	95	106	183	142	131	119	136	247	22
Complex IV	99 ± 9	119 ± 36	21 ± 4	23	5	38	31	28	12	18	360	12
Complex V	40 ± 4	14 ± 1	20 ± 9	8	75	32	20	9	6	8	130	0

Values for kidney and adrenal gland are given as mean +/- SD. [1]Enzymatic activities of kidneys have been reported previously (24). [2]Mean +/- SD of all cases excluding case 8.

Table 3. Scores for immunohistochemical staining of OXPHOS complexes and porin

	Adrenal gland (n=9)	Pheochromocytoma (n=10)	P value
Porin	197 ± 15	183 ± 15	0.4965
Complex I	125 ± 10	71 ± 22	0.0450
Complex II	135 ± 13	58 ± 12	0.0005
Complex III	198 ± 13	101 ± 21	0.0014
Complex IV	200 ± 13	115 ± 15	0.0006
ATP synthase	94 ± 10	73 ± 16	0.2853

Values are given as mean +/- SD. The score value is defined as the intensity of the staining multiplied with the percentage of positive cells.

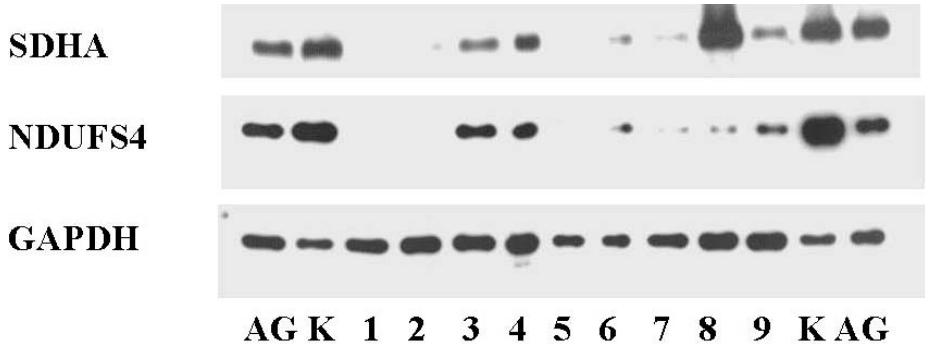

Figure 1. Western blot analysis of PC, kidney and adrenal gland tissue samples. Complex II (SDHA; subunit 70kDa Fp), GAPDH, VHL. K: normal kidney cortex tissue; AG: adrenal gland tissue; 1-9: PC samples.

Because only one of the analyzed PCs showed an isolated OXPHOS enzyme defect, we tested if the down-regulation of OXPHOS complexes is due to a general reduction of mitochondria. Therefore, we stained for the mitochondrial membrane protein porin, which can be used as marker for mitochondrial mass. The score values for porin revealed no significant difference between the PC tissues and adjacent adrenal gland (Table 3).

Detailed examination of the immunohistochemical staining at the cellular level revealed that sustentacular cells show strong positive staining for all OXPHOS complexes and porin. In contrast, a reduction or loss of the OXPHOS complexes is observed in the hormone-secreting cells (Figure 2 A - F).

VHL mutations are found in a subset of hereditary PCs. Because VHL is known to regulate degradation of hypoxia-inducible factor (HIF)-1α, which is a master regulator for induction of the glycolytic switch in tumor cells, we sequenced the VHL gene in seven of our sporadic PC cases but did not identify any pathogenic VHL mutations.

5. DISCUSSION

All but one of the nine investigated PCs showed a significant reduction in SDH activity compared to normal cortical kidney tissues. No LOH of SDH subunits was found in the examined samples, which indicates that the observed down-regulation of SDH activity in sporadic PC is not due to pathogenic mutations in genes encoding SDH. Our data are in agreement with those of Asturi *et al.*, who also did not find an association of sporadic PC with *SDHB* inactivation (25-27). Because those studies did not find inactivation of SDH genes by promoter methylation, we decided not to analyze the SDH genes in more detail.

Like complex II, complexes I, III and IV were also significantly reduced in sporadic PC while the tumor mitochondria showed normal amounts of porin. Therefore, down-regulation of the respiratory chain is not due to a more general decrease in mitochondrial mass. The expression of the OXPHOS complexes is significantly reduced in the hormone-secreting cells of the PC, whereas the sustentacular cells show a strong staining for all OXPHOS complexes (Figure 2). Because absence of S-100 positive sustentacular cells was demonstrated in the majority of malignant PCs (28), the low presence of these

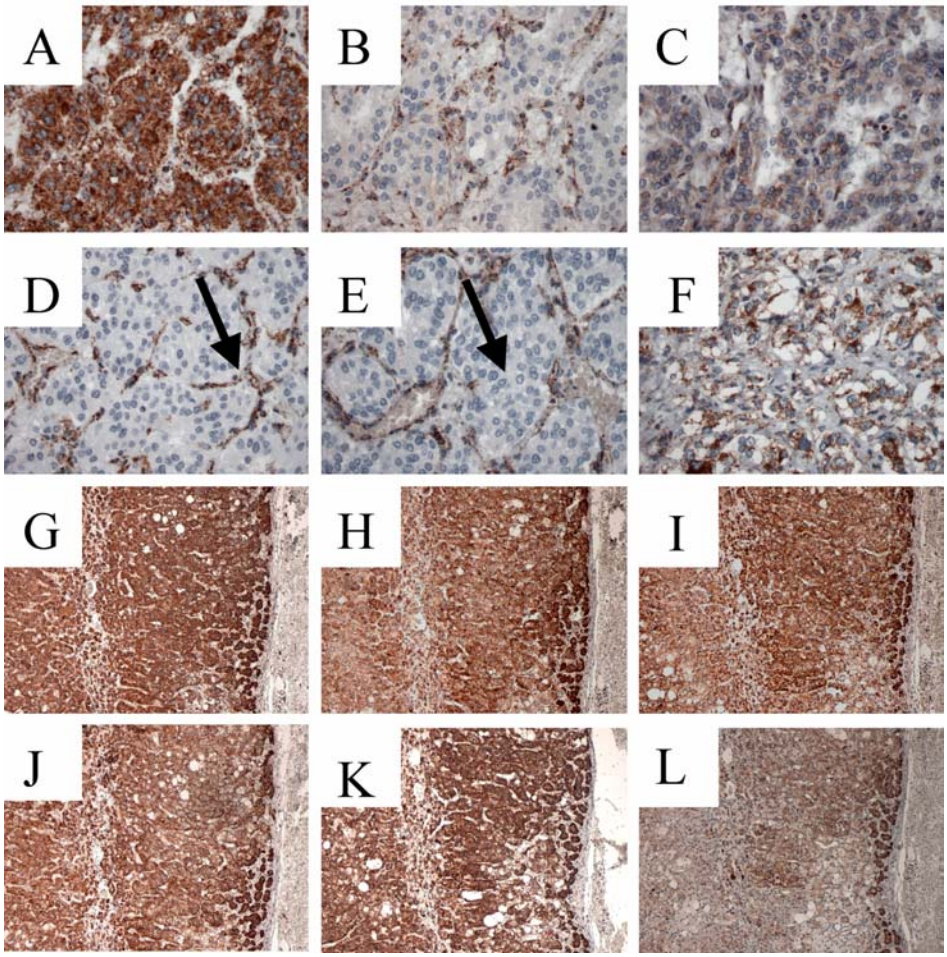

Figure 2. Immunohistochemical staining of porin (A, G), complex I subunit NDUFS4 (B, H), complex II subunit 70kDa Fp (C, I), complex III subunit Core2 (D, J), complex IV subunit I (E, K), and complex V subunit alpha (F, L) in unaffected adrenal gland tissue (G-L) was compared to the staining in adjacent PC tumor tissue (A-F). D: Arrow highlights positive sustentacular cells. E: Arrow highlights the negative chromaffin cells.

cells should not influence the overall metabolic phenotype of PC.

We performed an immunohistochemical analysis on normal adrenal tissue and were able to show that cells of the adrenal cortex and medulla, including chromaffin cells, have substantial staining of porin and OXPHOS complexes. This indicates clearly the down-regulation of OXPHOS complexes during tumor transformation and progression.

Although somatic inactivation of VHL occurs in most sporadic renal cell carcinomas it has been reported that this event is infrequent in sporadic PC (27). Accordingly, we could not detect VHL mutations in our samples. Therefore, VHL mutations can be excluded as being responsible for the low OXPHOS in our samples. Recently, it was shown that p53 gene mutations are frequently found in multiple and malignant PCs (29). p53 is an important regulator of mitochondrial energy metabolism and glycolysis (30). Lebedeva *et al.* found that p53 null mice and p53 knockdown human primary fibroblasts exhibit mitochondrial DNA depletion and reduced mitochondrial mass (31). However, this is unlikely to be the mechanism of OXPHOS down-regulation in the PCs analyzed in the present study, because we did not observe a substantial down-regulation of mitochondrial mass. The down-regulation of the OXPHOS complexes without reduction of mitochondrial mass has also been observed in undifferentiated NB, another neural crest-derived tumor (24).

One case stood out biochemically by exhibiting a phenotype characteristic for oncocytic tumors (Zimmermann 2010, this issue). Interestingly, histologically, no signs of oncocytic features could be found in this case (data not shown), which is in

contrast to what has been observed in other tumors with specific complex I deficiency and compensatory up-regulation of the citrate synthase and OXPHOS complexes II-V (Zimmermann 2010, this issue). This is, to our knowledge, the first case of a PC with a specific defect in complex I of aerobic energy metabolism.

In summary, in most sporadic PCs, low aerobic energy metabolism does not stem from a defect of a single complex of the respiratory chain, as it does in a subset of hereditary PCs. However, in the majority of sporadic PCs the activity of the respiratory chain on the whole is reduced. Notwithstanding the clinical uniformity of PCs, impairment of aerobic energy metabolism in PC seems to be achieved via a variety of routes, in view of the miscellaneous genetic and biochemical alterations found in hereditary and sporadic PCs.

6. ACKNOWLEDGMENTS

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