# Succinate Dehydrogenase (SDH) D Subunit (*SDHD*) Inactivation in a Growth-Hormone-Producing Pituitary Tumor: A New Association for SDH?

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**Background:** Mutations in the subunits B, C, and D of succinate dehydrogenase (SDH) mitochondrial complex II have been associated with the development of paragangliomas (PGL), gastrointestinal stromal tumors, papillary thyroid and renal carcinoma (SDHB), and testicular seminoma (SDHD).

**Aim:** Our aim was to examine the possible causative link between *SDHD* inactivation and somatotropinoma.

**Patients and Methods:** A 37-yr-old male presented with acromegaly and hypertension. Other family members were found with PGL. Elevated plasma and urinary levels of catecholamines led to the identification of multiple PGL in the proband in the neck, thorax, and abdomen. Adrenalectomy was performed for bilateral pheochromocytomas (PHEO). A GH-secreting macroadenoma was also found and partially removed via transsphenoidal surgery (TTS). Genetic analysis revealed a novel *SDHD* mutation (c.298_301delACTC), leading to a frame shift and a premature stop codon at position 133 of the protein. Loss of heterozygosity for the *SDHD* genetic locus was shown in the GH-secreting adenoma. Down-regulation of SDHD protein in the GH-secreting adenoma by immunoblotting and immunohistochemistry was found. A literature search identified other cases of multiple PGL and/or PHEO in association with pituitary tumors.

**Conclusion:** We describe the first kindred with a germline *SDHD* pathogenic mutation, inherited PGL, and acromegaly due to a GH-producing pituitary adenoma. *SDHD* loss of heterozygosity, down-regulation of protein in the GH-secreting adenoma, and decreased SDH enzymatic activity supports *SDHD*'s involvement in the pituitary tumor formation in this patient. Older cases of multiple PGL and PHEO and pituitary tumors in the literature support a possible association between SDH defects and pituitary tumorigenesis. **(*J Clin Endocrinol Metab* 97: E357–E366, 2012)**

Abbreviations: $C_T$, Cycle threshold; GHR, GH receptor; HIF-1$\alpha$, hypoxia-inducible factor-1$\alpha$; IHC, immunohistochemistry; LASMSA, long-acting somatostatin analog; LOH, loss of heterozygosity; MEN, multiple endocrine neoplasia; MRI, magnetic resonance imaging; PGL, paraganglioma; PHEO, pheochromocytoma; PRL, prolactin; SDH, succinate dehydrogenase; TSS, transsphenoidal surgery; WT, wild type.

Coexistence of pituitary adenomas and paraganglioma (PGL) or pheochromocytoma (PHEO) has not been recognized as a syndrome. We have identified 25 cases of acromegaly since 1964 copresenting with PHEO and one report of extraadrenal PGL and acromegaly (1). Such cases may represent a new multiple endocrine neoplasia (MEN) rather than a fortuitous coexistence.

Mutations in the subunits B, C, and D and recently in subunit A (*SDHB, SDHC, SDHD* and *SDHA*, respectively) of the succinate dehydrogenase (SDH) mitochondrial complex II are known to be associated with the development of PGL, PGL and gastrointestinal stromal tumors (Carney-Stratakis syndrome), as well as with renal, and papillary thyroid cancer, neuroblastoma, and adrenal medullary hyperplasia (2–12). A case of testicular seminoma has also been reported in association with *SDHD* mutation (13).

In the present report, we had the opportunity to study a unique family with multiple members affected by PGL caused by a novel *SDHD*-inactivating mutation. The proband presented with acromegaly, PHEO and PGL. Genetic studies in his GH-secreting adenoma showed loss of SDHD expression consistent with a possible tumor suppression function in the pituitary tumor of this patient.

## Subjects and Methods

### Clinical studies and tissue samples

The institutional review boards of the participating institutions have approved all studies. Blood and tissue samples were collected from the patient and the family members after informed consent was obtained. Tissues were collected at surgery and processed for routine histopathology and immunohistochemistry after formalin fixation and paraffin embedding.

### Hormonal assays

Plasma and urinary catecholamines and metanephrines were measured using standard assays, as described previously, at the National Institutes of Health Warren Magnuson Clinical Center and Mayo Clinical Medical Laboratories.

### DNA preparation and sequencing studies

DNA was extracted from peripheral blood leukocytes, frozen tissue samples, or cell lines according to manufacturer protocols (QIAGEN, Valencia, CA). Mutation analysis for exons and the surrounding intron boundaries was performed for *SDHB, SDHC, SDHD, MEN1, AIP,* and *CDKN1B* genes; the four exons of *SDHD,* eight exons of *SDHB,* six exons of *SDHC,* six exons of *AIP,* and two exons of *CDKN1B* were amplified and sequenced by PCR-based bidirectional Sanger sequencing. The primers used for *SDHB, SDHC, SDHD,* and *MEN1* have been described elsewhere (14, 15). The primers for *AIP* and *CDKN1B* mutation analysis are described in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). All amplified samples were ex-

amined by agarose gel electrophoresis to confirm successful amplification of each exon. Direct sequencing of the purified fragments was then done using the Genetic Sequencer ABI3100 Applied Biosystems (Foster City, CA) apparatus. Sequences were analyzed using Vector NTI 10 software (Invitrogen, Carlsbad, CA).

### Immunohistochemistry (IHC)

All IHC was performed in collaboration with Histoserve, Inc. (Germantown, MD) using standard procedures. Slides from the patient's pituitary tumor and PHEO were compared with those from a pituitary adenoma from a patient negative for any known mutations, from tissue from a normal pituitary gland and from sporadic PHEO. The following primary antibodies were used: SDHD, sc-67195, rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA); SDHB, HPA002868, rabbit polyclonal IgG (Sigma-Aldrich Inc., St. Louis, MO); and GH receptor (GHR) [N3C2], internal, GTX101192, rabbit polyclonal IgG (Gene Tex, Irvine, CA).

### Quantitative real-time PCR

*SDHD* loss of heterozygosity (LOH) was analyzed by quantitative real-time PCR using SYBR Green in an ABI 7700 Sequence Detection System (Applied Biosystems). The PCR cycling conditions were as follows: 2 min at 95 C, 40 cycles of 95 C for 15 sec and 60 C for 30 sec, and a final step at 72 C for 30 sec. The primer sequences for wild-type (WT) and mutant *SDHD* alleles are available in Supplemental Table 1. A cycle threshold ($C_T$) value in the linear range of amplification was selected for each sample in triplicate. *SDHD* dosage was determined using the $2^{-\Delta\Delta CT}$ method. The normalized value ($\Delta C_T$) for the WT and mutant *SDHD* allele in the tumor sample was then compared with the $\Delta C_T$ of both alleles in the peripheral DNA to produce a fold change ratio (normal dosage = 1).

### Immunoblotting

Western blot analysis was performed following standard procedures. Briefly, cells were lysed by homogenization in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitor cocktail I (EMD Biosciences, La Jolla, CA) with subsequent centrifugation at 10,000 rpm for 10 min at 4 C. Equal amounts of protein lysate were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies. The following antibodies were used for immunoblotting: SDHD, H00006392-M04, mouse monoclonal IgG (Novus Biologicals, Littleton, CO); SDHB, HPA002868, rabbit polyclonal IgG (Sigma-Aldrich Inc., St. Louis, MO), GHR [N3C2], internal, GTX101192, rabbit polyclonal IgG (Gene Tex); and hypoxia-inducible factor-1α (HIF-1α), NB100-479, rabbit polyclonal IgG (Novus Biologicals).

### Enzyme assay

For enzymatic assays, tissue homogenates were prepared using a mechanically driven (500 rpm/six strokes) Potter-Elvejhem homogenizer (Vineland, NJ) in 500 $\mu$l of an ice-cold solution consisting of 0.25 M sucrose, 20 mM Tris-HCl (pH 7.2), 2 mM EGTA, 40 mM KCl, and 1 mg/ml BSA. Activities of respiratory chain complexes were measured by spectrophotometer using a Cary 50 UV-visible spectrophotometer (Varian Inc., Les Ulis,
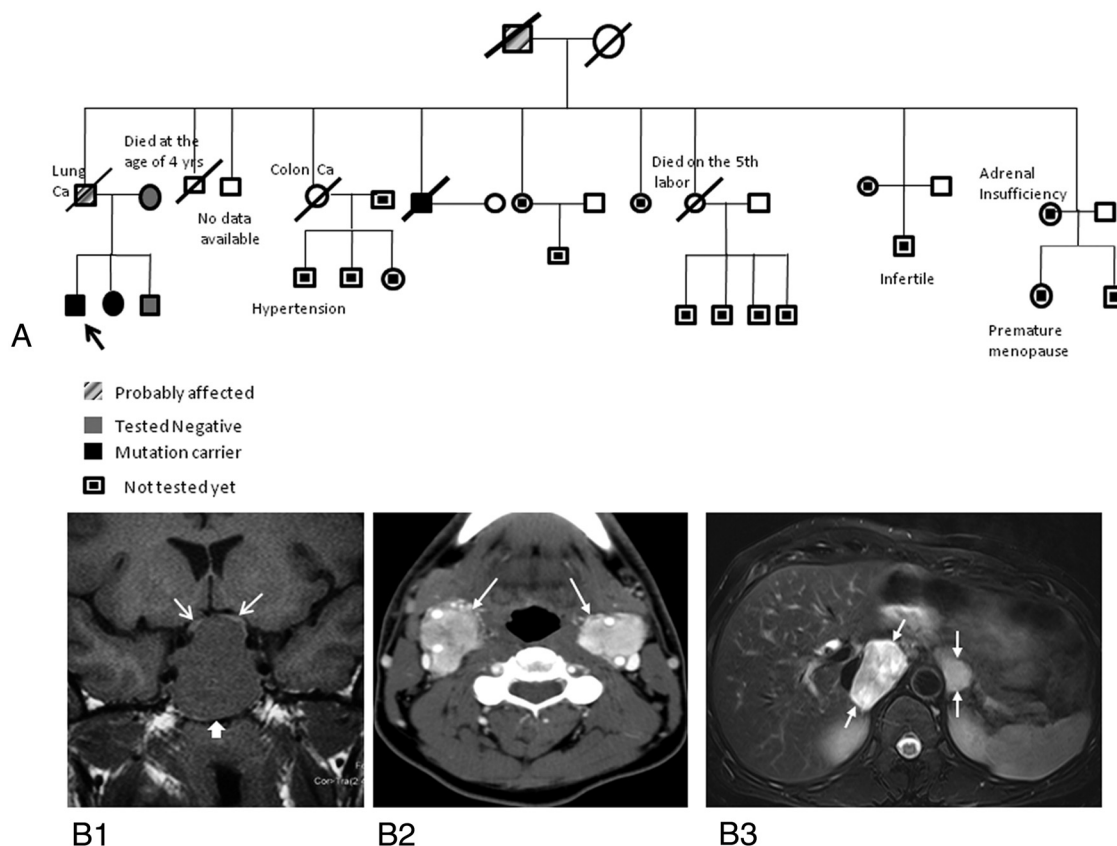
**FIG. 1.** A, Pedigree of the studied family. The *arrow* indicates the proband. B1, Coronal MRI scan of the pituitary gland without contrast. There is a large pituitary adenoma extending into the suprasellar region and compressing the optic chiasm (*long arrows*). There is evidence of invasion of both cavernous sinuses. The adenoma has also eroded the floor of the sella turcica and occupied the sphenoid sinus (*short arrow*). B2, Axial post-contrast computed tomography scan of the neck. Large enhancing tumors are identified in the soft tissues of the neck bilaterally (*arrows*). They are located at the bifurcation of the common carotid arteries between the proximal end of the internal and external carotids. The location of these tumors and the intense enhancement are typical for carotid body tumors. B3, Axial inversion recovery MRI scan of the upper abdomen with fat suppression. Tumor masses are identified in both adrenal glands with the right being larger than the left. Both tumors demonstrate high signal intensity with this technique (*arrows*). They are also intensely enhanced on the post-contrast scans (not shown). Histological examination of both tumors after complete surgical resection revealed PHEO.

France) as previously described (16, 17). All chemicals were from Sigma-Aldrich (St. Quentin, Falavier, France).

## Statistical analysis

All statistical analyses were performed with the SPSS version 16.0 (SPSS Inc., Chicago, IL). Continuous data are expressed as mean ± SE. A two-sample $t$ test was used for statistical analysis of gene dosage between WT and mutant alleles. A $P$ value < 0.05 was considered significant.

## Results

### Clinical course and investigation

The proband was a 41-yr-old man who was first evaluated at 37 yr for a pituitary mass detected on brain magnetic resonance imaging (MRI). He had a 3-yr history of hypertension controlled with calcium channel blockers, diuretics, and angiotensin II receptor inhibitors. The family history was remarkable for hypertension and type 2 diabetes in the patient's mother, whereas the patient's fa-

ther passed away from lung cancer at the age of 65 (Fig. 1A). The patient was clearly acromegalic upon presentation. Hormonal evaluation revealed GH of 10.70 ng/ml (normal is <5 ng/ml) and IGF-I of 492 ng/ml (normal is 109–284 ng/ml). Calcitonin and PTH were normal; serum prolactin (PRL) was mildly increased at 25.5 ng/ml (normal is <18); testosterone was decreased at 78 ng/dl (normal is 200–980 ng/dl). The patient had impaired fasting glucose according to standard criteria (18) and mild dyslipidemia with cholesterol at 225 mg/dl (normal is 130–200 mg/dl), high-density lipoprotein at 77 mg/dl (normal is >55 mg/dl), and low-density lipoprotein at 135 mg/dl (normal is <165 mg/dl). A pituitary MRI showed a 3.5- × 4.3- × 3-cm pituitary mass. The mass had invaded the right cavernous sinus and extended into the suprasellar region compressing the optic chiasm (Fig. 1B1).

The patient was placed on long-acting somatostatin analog (LASMSA) and showed a partial clinical and laboratory response (Table 1). The patient was scheduled for

**TABLE 1.** Clinical and biochemical response to treatment over time

| | Baseline | 3 months after LASMSA Rx | 6 months after LASMSA Rx | 12 months after LASMSA Rx | 24 months after LASMSA Rx (before TSS) | 3 months after TSS | 14 months after TSS | 26 months after TSS |
|---|---|---|---|---|---|---|---|---|
| GH (ng/ml) | 10.7 | 12.4 | 8.24 | 15.9 | 6.2 | 1.53 | 0.9 | |
| IGF-I (ng/ml) | 492 | 439 | 456 | 530 | 774 | 126 | 169 | 194 |
| Optic fields | Left eye nasal scotoma | Normal | Normal | Normal | Normal | Normal | Normal | Normal |
| Urine normetanephrine (μg/24 h) | | | | 3506 | 2220 | | 727 | 986 |
| Plasma normetanephrine (pg/ml) | | | | | 2396 | | 465 | 454 |

Rx, Treatment.

transsphenoidal surgery (TSS) after a year of treatment led to no improvements. Five tumors with characteristics typical for PGL were detected by computed tomography and MRI of the neck: two originating from the carotid bodies, two located in the jugular fossae, and one in the soft tissue at the carotid bifurcation (Fig. 1B2) and the base of the skull. There were also three masses in the thorax; one at the paraspinal region (fifth thoracic vertebra), one at the left pulmonary hilum, and the third at the epicardium. Two additional tumors were found in the retroperitoneum below the diaphragm and one in each adrenal gland (Fig. 1B3). [18F]Fluorodopa positron emission tomography imaging was also performed that showed avid concentration of the radionuclide in each of these tumors.

Plasma norepinephrine was 847 pg/ml (normal is 120–350 pg/ml), and urine normetanephrine was 3506 μg/24 h (normal is <500 μg/24 h). Plasma dopamine was also elevated at 153 ng/liter (normal is 30–120 ng/liter) as well as chromogranin A at 1087 ng/ml (normal is <98 ng/ml). Initially, the patient underwent bilateral adrenalectomy, and the diagnosis of PHEO was confirmed by histology. The pheochromocytoma of the adrenal gland scaled score (PASS) was 8, and Ki67 was 1%. Ten months later at the National Institutes of Health, just before TSS, plasma normetanephrine was 2396 pg/ml (normal is <112 pg/ml), 24-h urine normetanephrine was 2220 μg/24 h (normal is <419 μg/24 h), norepinephrine was 361 μg/24 h (normal is <80 μg/24 h), metanephrine was less than 29 μg/24 h, and IGF-I was 774 ng/ml (normal is <284 μg/24 h) (Table 1). The GHRH serum level was within the normal range at 9 pg/ml (normal is 5–18 pg/ml). The pituitary adenoma was partially resected. This somatotropinoma also stained positive for PRL; it was *P53* negative with a low proliferation index. He was placed on LASMSA and 3 months after TSS, the levels of IGF-1 and GH were normalized (Table 1). During his follow-up and while on LASMSA, a significant decrease in plasma and urine normetanephrine was detected (Table 1 and Fig. 2A). The size of the PGL was stabilized.

## Genetic and expression studies

The possibility of a hereditary syndrome was strongly suggested; family history revealed the case of a paternal uncle who had been operated 39 yr earlier for bilateral neck masses (Fig. 1A). Genetic analysis of the *SDHB*, *SDHC*, and *SDHD* genes revealed a novel *SDHD* exon 3 mutation (c.298_301delACTC), resulting in a frame shift and premature stop at codon 133. The same mutation was detected in the patient's affected relatives. The patient was also tested for genes *MEN-1*, *AIP*, and *CDKN1B*: he had no *MEN1* mutations, whereas one common polymorphism was detected in *CDKN1B* (c.326 T→G p.V109G) and another one in *AIP* (c.682 C→A, p.Q228K).

We then looked for *SDHD* LOH in tumor cells. Indeed, the *SDHD* WT allele was significantly reduced in the pituitary tumor in relation to the patient's peripheral blood (*SDHD* WT allele copy number at 0.3 ± 0.04 *vs.* 1.0 ± 0.01; $P < 0.0001$) (Fig. 3A). In addition, Western blot of extracts from the patient's pituitary tumor showed almost no expression of SDHD (Fig. 3B).

The 15-kDa band corresponding to the SDHD protein was present in normal pituitary as well as in extracts from pituitary tumor cells from another patient with acromegaly who did not have SDHx mutations (Fig. 3B). Consistent with the LOH and Western blot studies, IHC for SDHD was negative in the patient's pituitary tumor (Fig. 4.1) compared with that in normal pituitary cells (Fig. 4.2). Staining for SDHB showed diffuse but patchy staining in some areas of the tumor (Fig. 4.3); in other areas, SDHB staining was negative (Fig. 4.4). Normal pituitary showed also diffuse SDHB staining (Fig. 4.5) unlike what has been reported (19). We then looked for the expression
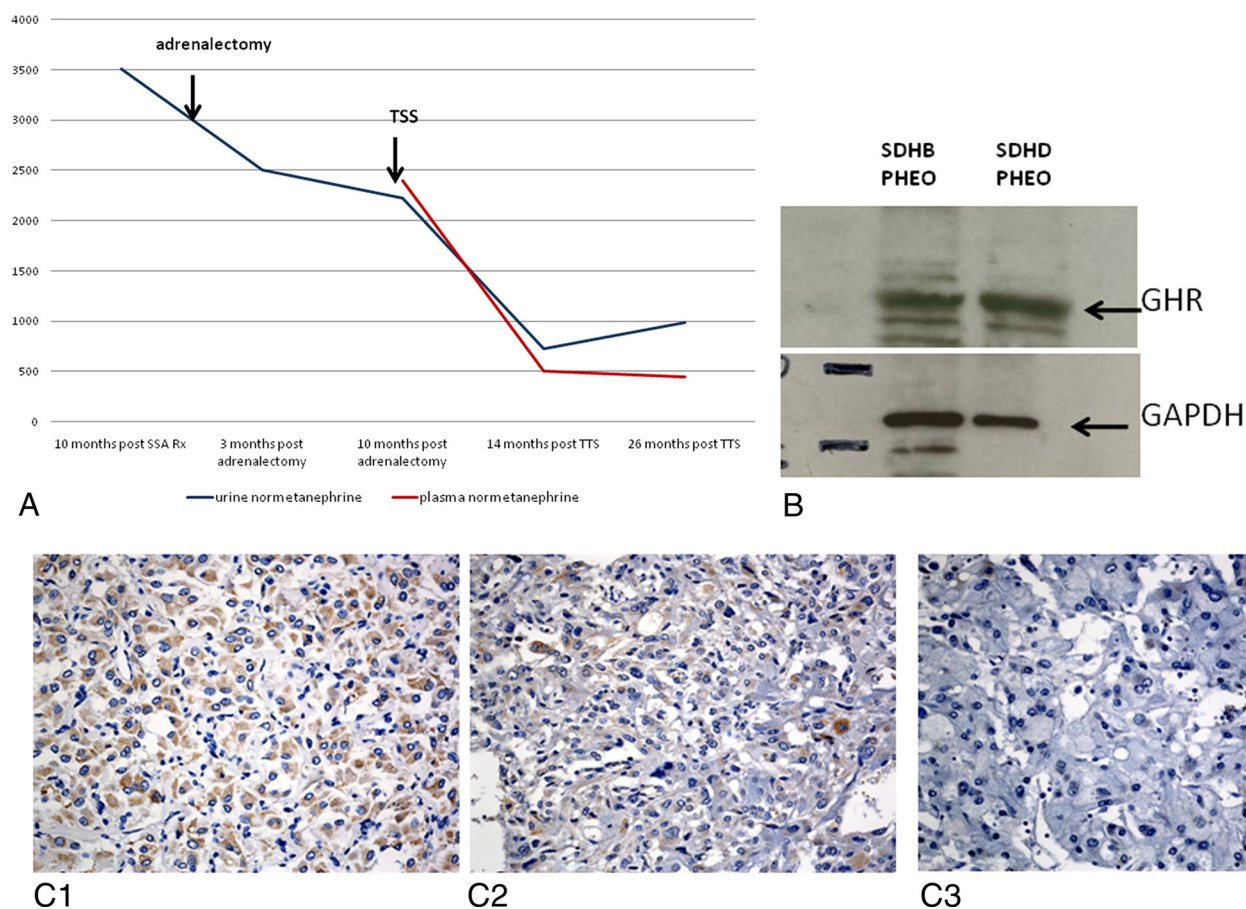
**FIG. 2.** A, Catecholamine levels over time; B, Western blot of extracts from PHEO harboring SDHx mutations showed a strong expression of GHR in both samples; C, staining for GHR was positive in our patient's resected PHEO (C1) and weak (C2) to almost negative (C3) in two sporadic adenomas. Original magnification, ×20.

of SDHB in the patient's pituitary tumor cells by Western blot. The 34-kDa band corresponding to the SDHB protein was weaker compared with the ones detected in extracts from normal pituitary as well as the GH-secreting adenoma without SDHx mutation (Fig. 3C). Staining for SDHB and SDHD in patient's PHEO was negative (Fig. 4.6 and 4.7). HIF-1α expression was found to be increased in our patient's tumor extracts compared with normal pituitary (Fig. 3C).

We also looked for any expression of GHR in extracts from PHEO from patients with *SDHB* and *SDHD* mutations and in paraffin-embedded tissue derived from our patient's resected PHEO and from two other patients with sporadic PHEO. Western blot of extracts from PHEO harboring SDHx mutations showed a strong expression of GHR: the 72-kDa band corresponding to the GHR protein was present in both samples (Fig. 2B). IHC for GHR showed positive diffuse staining in our patient's sample (Fig. 2C1). Staining was weaker in the two samples from sporadic PHEO without any known mutations (Fig. 2C2 and -3).

### Enzymatic activity

To assess the effect of the identified mutation on respiratory chain function, we performed enzymatic assays on homogenates prepared from the pituitary tumor tissue or control samples. The assay involved the measurement of the oxidation of added reduced cytochrome c (cytochrome oxidase activity) and subsequent reduction of added oxidized cytochrome triggered by succinate (complex II+III activity) or decylubiquinone (complex III activity). Upon addition of oxidized cytochrome c, before addition of any reducing substrate, a significant reduction of cytochrome c took place in the tumor homogenate, suggesting the presence of a significant amount of an endogenous reducing substance. Upon the subsequent addition of succinate, complex II substrate, increased rates of cytochrome c reduction in both set-ups were noted that were essentially sensitive to malonate, a specific inhibitor of complex II (Fig. 5). We next performed an isolated complex II activity assay using decylubiquinone (in the presence of dichlorophenol indophenol) as an electron acceptor. A 15–20% decrease in activity was measured in tumor tissue compared with control ($P < 0.08$).
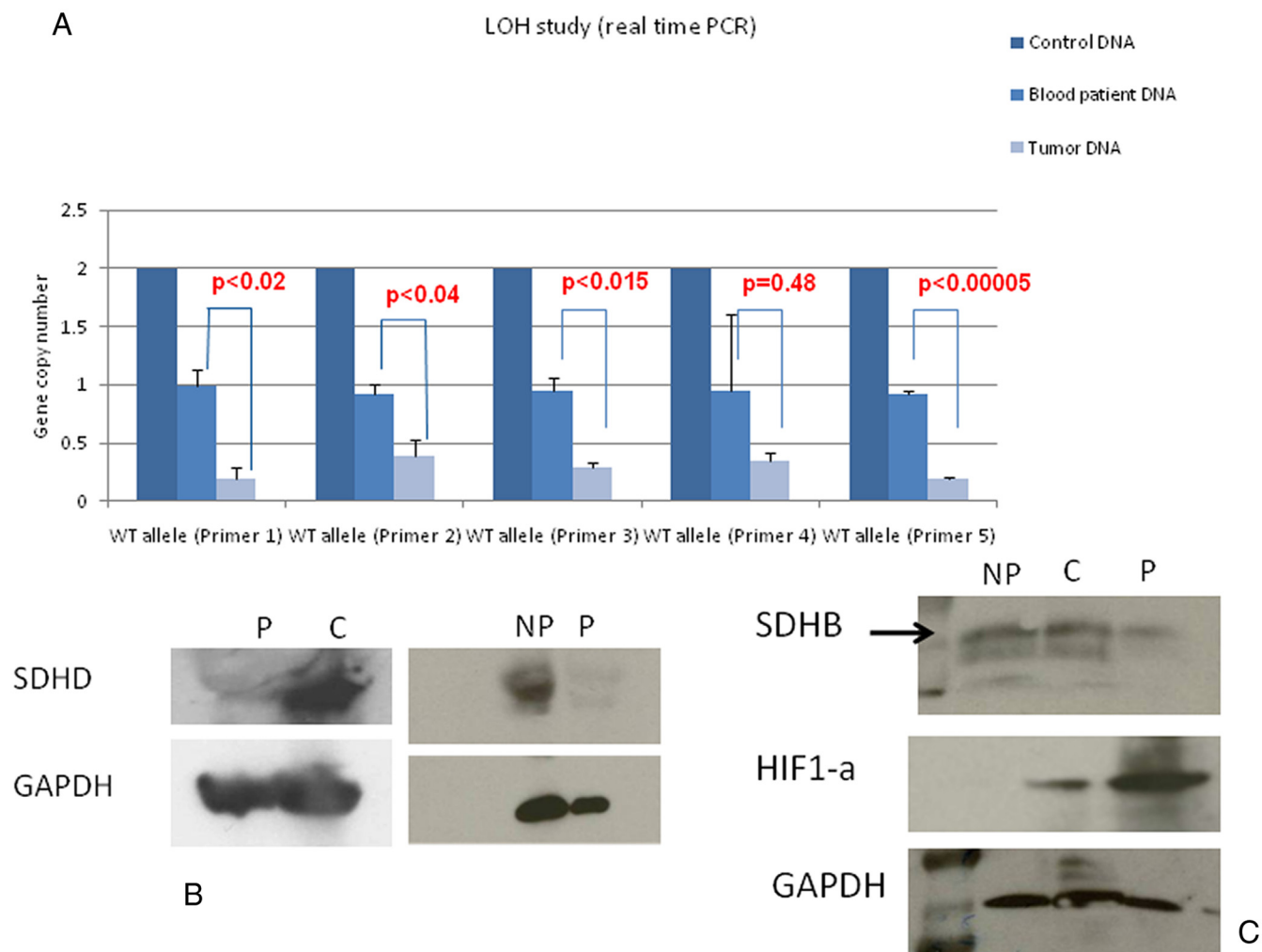
**FIG. 3.** Panel A, Relative expression of WT and mutant allele in the tumor and peripheral blood. Amplification of the *SDHD* WT allele was significantly reduced in the pituitary tumor in relation to the patient's peripheral blood (*SDHD* WT allele copy number, $0.3 \pm 0.04$ *vs.* $1.0 \pm 0.01$; $P < 0.0001$). Panel B, Western blot of SDHD expression in patient's tumor (P) and in normal pituitary (NP) as well as in extracts from pituitary tumor cells from a patient with acromegaly without SDHx mutations (C). Panel C, Western blot of SDHB expression in patient's pituitary tumor (P) was decreased, whereas HIF1α was significantly increased, compared with normal pituitary (NP) and with a patient with acromegaly without SDHx (C).

## Discussion

We describe for the first time the association of a novel germline *SDHD* pathogenic mutation with inherited PGL and a GH-producing pituitary adenoma. SDH participates in the electron transfer of the respiratory chain and in the conversion of succinate to fumarate as the complex II in oxidative phosphorylation as well as in the citric acid cycle (4, 20); its function and expression in the pituitary gland is as important as in all other tissues. It has been shown that SDH subunits act as tumor suppressor genes following the Knudson two-hit hypothesis (20, 21). The precise pathway leading from SDH mutation to tumorigenesis is not yet fully elucidated, but one of the mechanisms proposed is through accumulation of HIF-1α and -2α (22–25). SDH-inactivating mutations create a pseudo-hypoxia state that is characterized by succinate and reactive oxygen species accumulation, resulting in increased HIFα levels that in turn activate oncogenesis (20, 22–27). Indeed, HIF1α immunoblotting in our patient's tumor showed increased expression compared with a GH-secreting adenoma without SDH defects and with normal pituitary (Fig. 3C). Impaired oxidative phosphorylation forces the tumor cell to create ATP glycolytically even during normoxia, the so-called Warburg effect (22).

The structure of mitochondrial complex II or succinate-ubiquinone oxidoreductase (mitochondrial SQR) contains four proteins: flavin adenine dinucleotide-binding protein or flavoprotein (Fp or subunit A), iron-sulfur protein (Ip or subunit B), and two membrane-anchor proteins (CybL or subunit C and CybS or subunit D) with a total of six transmembrane helices. Subunits A and B form the soluble catalytic heterodimer, whereas subunits C and D
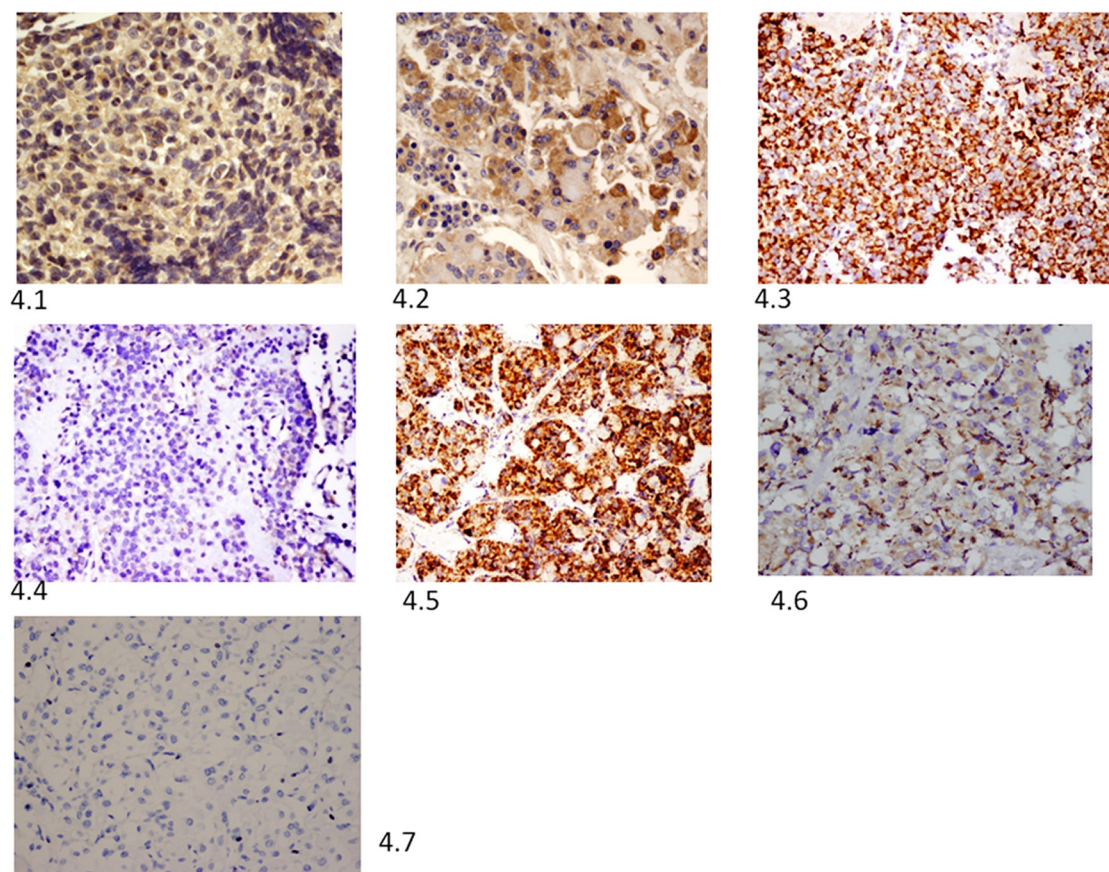
**FIG. 4.** IHC showed loss of expression of *SDHD* protein (4.1) compared with normal pituitary (4.2). SDHB staining was positive for part of the tumor (4.3) whereas in other parts was totally negative (4.4). Normal pituitary showed strong diffuse SDHB staining (4.5). Patient's PHEO was stained negative for both SDHB and SDHD (4.6 and 4.7) Original magnification, ×20 (4.1, 4.2, 4.6, and 4.7) and ×100 (4.3–4.5).

form the hydrophobic membrane anchor (28). Subunit D has four helices: helix 1S (D36–D62), helix 2S (D66–D92), helix 3S (D95–D123), and helix 4S (D126–D136) (27). There are at least two ubiquinone-binding sites in eukaryotic mitochondrial succinate-ubiquinone oxidoreductase. One site (Qp) is on the matrix side, and the second (Qd) is near the intermembrane-space side. The latter is formed by the loop between the helix 1S and helix 2S and helix 4S and the C terminus of CybL (29). The mutation reported in the present case is a 4-bp deletion (ACTC) at position c.298_301, which, based on the described structure, would result in missing both helices 3S and 4S of subunit D. This most probably would prevent ubiquinone from binding to the Qd site, disrupting the transportation of the high-energy electrons through the respiratory chain.

Since the discovery of the SDHx genes (30) and its relation to familial PGL/PHEO, other forms of tumors associated with inactivating SDH subunit mutations have been described in recent years in gastrointestinal stromal tumors and in Carney-Stratakis syndrome (2, 3), in renal cancer (10, 11), in patients with *PTEN*-negative Cowden and Cowden-like syndrome (7), in thyroid cancer (10),

and in patients with adrenal medullary hyperplasia (12). We were able to identify 25 cases of pituitary adenoma and PHEO in the literature (1). The pituitary tumors have included GH-, PRL-, and ACTH-secreting and nonfunctioning adenomas.

Given these data, we examined whether the pituitary tumor in our kindred was due to the *SDHD* mutation. The patient did not have any mutations in *MEN1*, *AIP*, and *CDKN1B* genes. Because *SDHD* acts as tumor suppressor gene, we examined whether LOH was present in the patient's pituitary tumor. Indeed, the mutated allele was primarily expressed in the pituitary tumor, whereas both alleles were equally expressed in the peripheral blood. In fact, the detection of any expression of the normal allele in the pituitary tumor was most likely due to contamination by normal cells as often is the case in tissue lysates (31). SDHD expression by Western blot and IHC was decreased in the patient's pituitary tumor, compared with a GH-secreting adenoma negative for *SDHD* mutation and with normal pituitary. SDHB expression was also significantly reduced by immunoblotting (Fig. 3C). SDHB staining was positive for parts of the pituitary tumor (Fig. 4.3), whereas other parts of the tumor were completely negative (Fig.
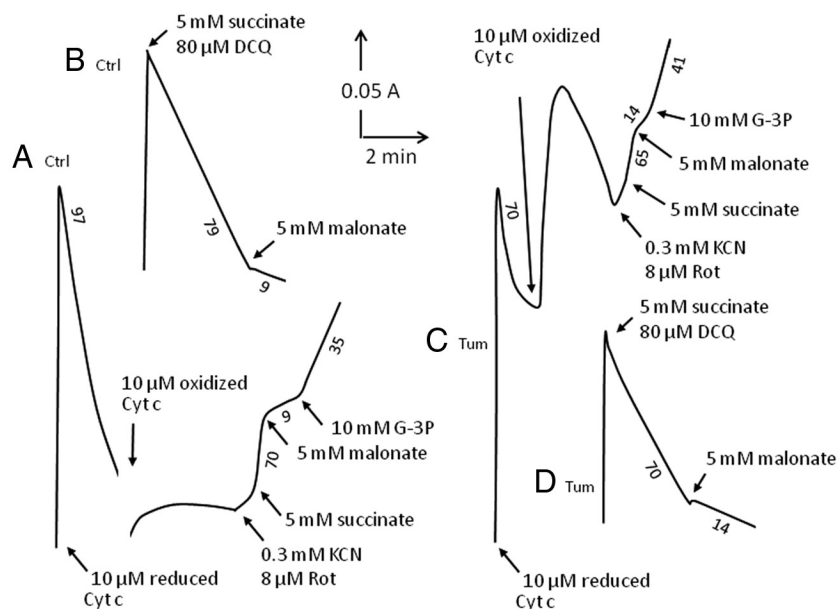
**FIG. 5.** Respiratory chain activity in control (Ctrl) and tumor (Tum) tissues samples. A and B, Cytochrome c oxidase (complex IV) activity is first measured, triggering the oxidation of the added reduced cytochrome c (Cyt c). After addition of oxidized cytochrome c and the subsequent inhibition of complex IV by cyanide (KCN) and complex I by rotenone (Rot), the activity of the succinate cytochrome c reductase (complex II+III) is initiated by the addition of succinate and specifically inhibited by malonate. The final addition of glycerol-3 phosphate (G-3P) permits estimation of the activity of the G-3P cytochrome c reductase (G-3P dehydrogenase+complex III). Notice that in the tumor sample (B) compared with control (A) a spectacular reduction of the oxidized cytochrome c added after the measurement of the cytochrome c oxidase took place, indicating the huge accumulation of reducing compounds in the tumor tissue (A). C and D, Measurement of the malonate-sensitive succinate quinone reductase (complex II; succinate dehydrogenase) activity in control (C) and tumor (D) samples. Assay medium was initially supplemented with 80 $\mu$M dichlorophenol indophenol and 0.3 mM KCN; the reaction is initiated by the addition of succinate and decylubiquinone (DCQ) and inhibited by the addition of malonate. *Numbers along the traces* are nanomoles per minute per milligram protein. Experimental conditions are as described in *Materials and Methods*.

4.4). The patient's PHEO was negative for SDHB and SDHD (Fig. 4.6 and 4.7), which is in accordance with van Nederveen *et al.* (19) who have shown that SDHB staining is absent in all tumors harboring an *SDHx* mutation. However, in their paper, Gill *et al.* (32) showed that completely absent staining is more commonly found in tumors with *SDHB* mutation, whereas weak diffuse staining often occurs in those with *SDHD* mutation. Our findings in pituitary tumor agree with Gill *et al.* (32); the SDHD-deficient pituitary tumor had some staining for SDHB unlike what would be expected from an *SDHB*-mutant lesion. In addition, SDHB staining was patchy; some areas had relatively strong staining for SDHB, others less, and others were completely negative. It is unclear what this patchy staining is from; the genetic data and allelic heterogeneity supported a possible polyclonal origin or substantial contamination with normal cells. Unless one had access to cells obtained by microdissection from well-defined areas of the tumor, the above conundrum will remain unanswered.

We also measured the SDH enzymatic activity in a tissue sample from normal pituitary as well in a tissue sample from our patient's pituitary adenoma. It is interesting that initially, when we added oxidized cytochrome c, a significant reduction of cytochrome c took place in the tumor homogenate, suggesting the presence of a significant amount of one or more endogenous reducing substances (Fig. 5). This could represent either reducing acids resulting from SDH impairment or oxidatively modified compounds resulting from unstable reactive oxygen species, possibly produced as a result of SDHx mutations (23). When we measured the activity of SHD alone, a 15–20% reduction was found in tumor tissue compared with control ($P < 0.08$). One possible explanation for the fact that the SDH activity was not completely abolished in the tumor may be that the homogenate contained normal cells, which compensated for the reduced activity of the tumor cells. Unfortunately, we did not have enough tumor cell lines with homogeneous cell populations to answer these questions properly.

Studies in family members (Fig. 1) did not reveal any pituitary tumors. Whether carriers develop the tumor depends on many factors (33); in addition, the age-related penetrance *of SDHD*-linked PGL is 54% by the age of 40 yr and 68% by the age of 60 yr, reaching a maximum of 87% by the age of 70 yr (34). Review of the other cases reported in the literature did not reveal the presence of PGL and pituitary adenomas in other family members. Genetic analysis was not performed in most cases, except for *RET* (35, 36). It is also possible that some of these cases are due to GHRH-secreting neuroendocrine tumors (37, 38).

Another interesting finding in our index case was a noticeable decrease (almost three times) of plasma and urinary norepinephrine and normetanephrine levels 14 and 26 months after TSS, which was greater than the one noted 10 months after adrenalectomy (Fig. 2A). No progression in the size of the multiple PGL was noticed. One possibility is that LASMSA had an effect on catecholamine release; however, the decline was not seen before TSS despite the patient being on LASMSA for 10 months, and it was sustained after TSS even when the patient stayed off LASMSA

for brief periods of time. Thus, we assumed that normalization of GH levels after TSS contributed significantly to such biochemical changes. We showed that GHR was expressed in two PHEO samples from other patients harboring each an *SDHB* and an *SDHD* mutation, respectively, as well as in the PHEO from our patient. As far as we know, this is the first time that the expression of GHR is shown in PHEO. There are only reports for the differential expression of ghrelin and GHRH receptors in various adrenal tumors (PHEO included) (39, 40). Clearly, larger and more detailed studies are needed to address and confirm this very interesting observation and the role of GHR in SDHx-mutant tumors.

In conclusion, we have identified a novel *SDHD* mutation in a patient with multiple functioning PGL and a pituitary adenoma. Whether the SDH genes are indeed predisposing genes for pituitary tumors requires additional studies; this report only suggests that a careful family history regarding pituitary tumors should be considered in patients with multiple PGL or with any germline genetic defects of the SDH genes.

## Acknowledgments

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