SDHA is a tumor suppressor gene causing paraganglioma

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Mitochondrial succinate-coenzyme Q reductase (complex II) consists of four subunits, SDHA, SDHB, SDHC and SDHD. Heterozygous germline mutations in SDHB, SDHC, SDHD and SDHAF2 [encoding for succinate dehydrogenase (SDH) complex assembly factor 2] cause hereditary paragangliomas and pheochromocytomas. Surprisingly, no genetic link between SDHA and paraganglioma/pheochromocytoma syndrome has ever been established. We identified a heterozygous germline SDHA mutation, p.Arg589Trp, in a woman suffering from catecholamine-secreting abdominal paraganglioma. The functionality of the SDHA mutant was assessed by studying SDHA, SDHB, HIF-1α and CD34 protein expression using immunohistochemistry and by examining the effect of the mutation in a yeast model. Microarray analyses were performed to study gene expression involved in energy metabolism and hypoxic pathways. We also investigated 202 paragangliomas or pheochromocytomas for loss of heterozygosity (LOH) at the SDHA, SDHB, SDHC and SDHD loci by BAC array comparative genomic hybridization. In vivo and in vitro functional studies demonstrated that the SDHA mutation causes a loss of SDH enzymatic activity in tumor tissue and in the yeast model. Immunohistochemistry and transcriptome analyses established that the SDHA mutation causes pseudo-hypoxia, which leads to a subsequent increase in angiogenesis, as other SDHx gene mutations. LOH was detected at the SDHA locus in the patient’s tumor but was present in only 4.5% of a large series of paragangliomas and pheochromocytomas. The SDHA gene should be added to the list of genes encoding tricarboxylic acid cycle proteins that act as tumor suppressor genes and can now be considered as a new paraganglioma/pheochromocytoma susceptibility gene.
INTRODUCTION

Paraganglioma and pheochromocytoma are rare tumors of chromaffin tissue that may secrete catecholamines. They arise in the adrenal medulla (pheochromocytoma proper) or in extra-adrenal regions in the thorax, abdomen or pelvis. They may also be derived from parasympathetic tissue of the head and neck. Paragangliomas and pheochromocytomas occur either sporadically or in the context of several inherited syndromes: multiple endocrine neoplasia type 2, von Hippel–Lindau disease, neurofibromatosis type 1 and familial paraganglioma-1 (PGL1), PGL2, PGL3 or PGL4 caused, respectively, by germ-line mutations in the RET, VHL, NF1 and SDHD, SDHAF2, SDHC or SDHB genes (1,2). SDHD, SDHB and SDHC encode three of the four subunits of succinate-coenzyme Q reductase (complex II, succinate dehydrogenase, SDH), a mitochondrial enzyme located at the crossroads between the tricarboxylic acid (TCA) cycle and the respiratory chain. SDH catalyzes the oxidation of succinate to fumarate and transfers electrons directly to the ubiquinone pool. SDHC (SDHx) mutations cause a cascade of molecular events leading to the abnormal stabilization of hypoxia-inducible factors (HIF) under normoxic conditions (2) or pseudo-hypoxia (via inactivation of SDH, accumulation of succinate, inhibition of proton-4-hydroxylases and subsequent impairment of HIF hydroxylation) (3,4), thereby promoting cell proliferation, angiogenesis and tumorigenesis (5).

Mutations in SDHA, which encodes the fourth subunit of SDH, have never been described in hereditary paraganglioma/pheochromocytoma. Biallelic SDHA mutations have been shown to cause an early onset encephalopathy known as Leigh syndrome (6–9). Reports of patients with complex II deficiency but lacking mutations in any of the four SDH genes suggested the existence of additional nuclear genes involved in the synthesis, assembly or maintenance of SDH (8). This was confirmed by the recent description of homozygous mutations in the SDHAF1 (SDH assembly factor 1) gene, causing infantile leukoencephalopathy (10). A mutation in another gene, SDHAF2, involved in the assembly of SDH was also recently reported. Mutations in SDHAF2, encoding the SDH assembly factor 2, required for SDH activity and stability, were described in two families affected by head and neck paragangliomas (2,11). SDHA, the flavoprotein-containing subunit of SDH, contains a covalently attached flavin adenine dinucleotide (FAD) cofactor. The requirement of SDHAF2 for flavination is supported by a dramatic decrease in FAD in SDHA with a concomitant loss of SDH enzymatic activity in SDHAF2-related paragangliomas (2).

It has been a source of puzzlement that while paraganglioma/pheochromocytoma has been associated with mutations in SDHB, SDHC, SDHD and most recently in a gene involved in flavination of SDHA, none have been reported in SDHA. On the basis of the previous claim of the existence of two human SDHA isoforms encoded by two distinct genes (12), a possible explanation for the absence of SDHA-related paraganglioma/pheochromocytoma was that it would require tetra-allelic genetic events in two independent SDHA loci (3,12,13). This explanation, however, lost credibility in view of later evidence favoring the existence of a single highly polymorphic SDHA gene (14).

Here, based on the observation of a patient with an extra-adrenal paraganglioma resulting from a loss-of-function germ-line mutation in SDHA, we show that SDHA, like other SDHx genes, can act as a tumor suppressor gene and activate the pseudo-hypoxic pathway.

RESULTS

Identification of the SDHA mutation and loss of heterozygosity at the SDHA locus

Genetic testing was proposed to the patient affected by an extra-adrenal paraganglioma in accordance with the international recommendations (15).

Mutation analyses in RET, VHL, SDHB, SDHC and SDHAF2 genes were negative. Enzyme assays were performed on tumor tissue. Succinate cytochrome c reductase activity (complex II + III) measured in tumor homogenate indicated a total and selective loss of SDH activity (Supplementary Material, Fig. S1). At the same time, the tumor was added to a series of paraganglioma/pheochromocytoma transcriptome analyses as described previously (16). Unsupervised analysis of 103 genes’ expression involved in energy metabolism (oxidative phosphorylation and glycolytic pathways) classified the patient’s tumor in the subgroup of SDH-related paraganglioma/pheochromocytoma (Fig. 1). These data strongly suggested a defect in one of the SDHx genes. Sequence analysis was thus extended to SDHA.

A missense mutation (c.1765C>T; p.Arg589Trp) was identified in germline DNA as well as in DNA and mRNA extracted from the tumor (Fig. 2A). Arginine 589 occurs in a highly conserved sequence of eu- karyotic and prokaryotic SDHs, including Saccharomyces cerevisiae (Fig. 2B). The c.1765C>T variant was not found in 740 control chromosomes and in silico predictions indicated structural alterations in the protein as a result of the mutation. The predominance of the W589 mutant allele in tumor DNA (Fig. 2A, middle panel) and cDNA (Fig. 2A, right panel) suggested loss of heterozygosity (LOH) at the SDHA locus. By BAC array comparative genomic hybridization (CGH), we analyzed the LOH pattern in the patient’s tumor included in a series of 202 paragangliomas and pheochromocytomas collected by the COMETE network. For the patient’s tumor, we observed a 5p14–5p15 loss, confirming the LOH at the SDHA locus (Fig. 2C). The LOH pattern in the complete series showed that loss of 5p15 region (SDHA locus, 9p21, 4.5%) and 1q21 (SDHC locus, 9q22, 4.5%) are frequent events compared with 1p36.1 (SDHB locus, 131/202, 64.9%) and 11q23 (SDHD locus, 55/202, 27.2%) losses (data not shown). The 9/202 tumors harboring a 5p15 loss included the patient’s tumor, two NF1-, one VHL-, one RET-, one SDHB-, one SDH-related paraganglioma/pheochromocytoma and two apparently sporadic tumors. No SDHA mutation was found in these two last samples.

The human SDHA is a unique and highly polymorphic gene

Two distinct tissue-specific human SDHA cDNAs differing in a few nucleotides leading to two amino acid changes, ‘type I Fp’ (Y629 and V657) and ‘type II Fp’ (F629 and I657), were
reported in 2003 (12). Subsequently, Baysal et al. (14) attributed the sequence differences to polymorphisms and concluded that only one SDHA gene exists in humans. To address the issue of two different SDHA genes, we analyzed SDHA cDNAs in different tissues. The tumor tissue of the patient contained only ‘type II Fp’ (F629 and I657). As we had previously observed the simultaneous presence of ‘type I Fp’ and ‘type II Fp’ in one pheochromocytoma sample (3), we extracted RNA from two additional pheochromocytomas and four fibroblast cultures from healthy control individuals. Y629 associated with V657 (corresponding to ‘type I Fp’) were found in three out of the four cultured fibroblasts and in the two pheochromocytoma controls. The fourth cultured fibroblasts contained both types (‘I Fp + II Fp’). Finally, we sequenced nucleotides at positions 1680, 1752, 1886, 1932 and 1969 (described as difference between type I Fp and type II Fp) of SDHA in germline DNA of 216 healthy control subjects. For each of the five positions, two different nucleotides were observed. Haplotype analysis using the Thesias program showed six rare haplotypes and two common haplotypes: 1680G-1752A-1886A-1932G-1969G, corresponding to ‘type I Fp’ (f = 0.817) and 1680A-1752G-1886T-1932A-1969A to ‘type II Fp’ (f = 0.125) (Table 1). Strong linkage disequilibrium was identified among the five single-nucleotide polymorphisms with all the D' values falling between 0.94 and 1.00 (P < 0.001). Together, these data constitute compelling evidence that SDHA, in agreement with the conclusions of Baysal et al. (14), is a unique polymorphic gene.

The SDHA mutation abolishes SDH activity in a yeast model

To assess the function of the mutant protein in vitro, we expressed the Arg589Trp in yeast. Arginine 582 of yeast Sdh1 protein corresponds to arginine 589 of human SDHA. This codon was changed to a tryptophan codon in the yeast SDH1 gene. The growth defect of the yeast SDH1 null mutant on a minimal ethanol/glycerol medium was not rescued by a chromosomally integrated copy of the Arg582Trp mutant gene (aW303ΔSDH1/ST19; Fig. 3A). In contrast, the null mutant transformed with the wild-type (WT) gene in the same vector (aW303ΔSDH1/ST20) grew as well as the WT on this medium. The inability of the mutant gene to restore SDH activity in the SDH1 null mutant (Table 2) confirmed Arg589Trp to be a loss-of-function mutation. As expected, integration of the WT gene in the SDH1 mutant restored enzyme activity to nearly WT levels.

The steady-state abundance of SDH in the WT and mutant strains, assessed by western analysis of total mitochondrial proteins (Fig. 3B), indicated comparable amounts of Sdh1 in mitochondria of WT and the transformant with the WT gene. No Sdh1 was detected in the SDH1 null mutant and the mutant transformed with the Arg582Trp gene (Fig. 3B). Western analysis also failed to reveal the presence of Sdh1 in the cytosolic fraction of the four different strains. These results indicate that the structural change resulting from the Arg582Trp mutation renders the protein more susceptible to proteolysis.

The SDHA mutation leads to SDHA and SDHB protein losses in tumor tissue

In order to confirm the instability of the mutant SDHA protein in human, we performed immunohistochemical analysis of the protein in the patient’s tumor. Strong SDHA immunostaining was observed in the cytoplasm of tumor and endothelial cells in all paraganglioma/pheochromocytoma tissues used as controls (RET, NF1, SDHB and SDHD), but was only present in the endothelial cells and was not detected in tumor cells of the patient.
As recently reported for all SDHx-related tumors (17), we did not detect SDHB immunostaining in the SDHA-mutant tumor (Fig. 4). In contrast, COX-IV protein expression, used as an internal control, was positive in all samples. The SDHA mutation causes pseudo-hypoxia. Pseudo-hypoxia is a known consequence of SDHB and SDHD mutations. We therefore compared HIF-1α expression by immunohistochemistry on the patient’s tumor and on RET, SDHB and SDHD (data not shown) paraganglioma or pheochromocytoma tissues. HIF-1α was highly expressed in the SDHA- as well as in the SDHB- and SDHD-related tumors (Fig. 5A). In contrast, it was undetectable in the RET tumor. We also obtained expression data for 54 HIF target genes using transcriptome analysis. The expression profile of the corresponding probe sets was used to perform a clustering on 69 RET-, NF1-, SDH- and VHL-related tumors, including that of the patient. The unsupervised analysis using the hypoxic pathway enabled us to classify the tumors into two

Table 1. Genotype repartition and allelic frequencies of SDHA nucleotides 1680, 1752, 1886, 1932 and 1969 in a control population

<table>
<thead>
<tr>
<th>SDHA exon</th>
<th>Exon 13</th>
<th>Exon 14</th>
<th>Exon 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotide position</td>
<td>c.1680</td>
<td>c.1752</td>
<td>c.1886</td>
</tr>
<tr>
<td>Genotype repartition in the control population (n = 216)</td>
<td>GG: 66%</td>
<td>AA: 67%</td>
<td>AA: 68%</td>
</tr>
<tr>
<td>GA: 31%</td>
<td>GA: 31%</td>
<td>AT: 30%</td>
<td>AG: 29%</td>
</tr>
<tr>
<td>AA: 3%</td>
<td>GG: 2%</td>
<td>TT: 2%</td>
<td>AA: 3%</td>
</tr>
<tr>
<td>Allelic frequencies</td>
<td>G&lt;sup&gt;a&lt;/sup&gt;: 81.9% (354/432)</td>
<td>A&lt;sup&gt;a&lt;/sup&gt;: 82.9% (358/432)</td>
<td>A&lt;sup&gt;a&lt;/sup&gt;: 83.3% (360/432)</td>
</tr>
<tr>
<td>A&lt;sup&gt;a&lt;/sup&gt;: 18.1% (78/432)</td>
<td>G&lt;sup&gt;b&lt;/sup&gt;: 17.1% (74/432)</td>
<td>T&lt;sup&gt;b&lt;/sup&gt;: 16.7% (72/432)</td>
<td>A&lt;sup&gt;b&lt;/sup&gt;: 17.4% (75/432)</td>
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*aNucleotide reported to be present in ‘type I Fp’.

*bNucleotide reported to be present in ‘type II Fp’.

(Fig. 4). As recently reported for all SDHx-related tumors (17), we did not detect SDHB immunostaining in the SDHA-mutant tumor (Fig. 4). In contrast, COX-IV protein expression, used as an internal control, was positive in all samples.

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The SDH and succinate cytochrome c reductase (SCCR) activities are expressed, respectively, as nmol of DCPiP and cytochrome c reduced min⁻¹ (mg of mitochondrial protein)⁻¹. After 36 h of growth in liquid YPGal, the SDH1 mutant and the transformant with the Arg582Trp mutation accumulated ≏50% secondary r and o mutants lacking part or all their mitochondrial genome. Under similar conditions of growth, there were <2% r and o mutants in the WT and the transformant with the WT gene. The instability of mitochondrial DNA in the mutant is not surprising as it has also been reported in several TCA cycle mutants such as aconitase, isocitrate dehydrogenase and pyruvate dehydrogenase-deficient strains (36).

The SDH and NF1 tumors and the second one included all VHL- and SDHx-mutated paragangliomas and pheochromocytomas, including the SDHA tumor (Fig. 5B).

Finally, we used CD34 immunohistochemistry to evaluate angiogenesis in the patient’s tumor (Fig. 5A). We observed that vascular density in the SDHA tumor (34 ± 3 blood vessels/0.65 mm²) was comparable to that observed in eight SDHx-related tumors (mean = 31 ± 4 blood vessels/0.65 mm²) and higher than in five RET/NF1 tumors (mean = 9 ± 2 blood vessels/0.65 mm²), which we recently reported (16). All these data suggest that SDHA inactivation stimulates
angiogenesis via the pseudo-hypoxic pathway as previously described for the other SDH subunits.

DISCUSSION

The observation of a patient with a paraganglioma resulting from a germline mutation in SDHA (p.Arg589Trp) associated with LOH in tumor provides the first evidence that mutations in SDHA can cause paraganglioma and that SDHA, like other SDHx genes, can act as a tumor suppressor gene in accordance to the Knudson’s ‘two-hits’ model.

Immunohistochemistry revealed that SDHA was absent from tumor cells of the patient with the p.Arg589Trp mutation. This suggested that the p.Arg589Trp mutation leads to SDHA protein instability, a conclusion supported by the results obtained with the yeast model. Introduction of the homologous Arg582Trp in the Sdh1 subunit of the yeast complex abolished SDH activity with a concomitant reduction of mitochondrial Sdh1 to undetectable levels. This putative instability of the Trp589 SDHA is consistent with the amino acid substitution and its location in the protein. Arg589 is located at the apex of SDHA (18,19), about 20 Å removed from the flavin moiety and distal to the interface with SDHB (>30 Å) (Fig. 3C). This makes it unlikely that Arg589 plays a direct role in the activity or assembly of the complex. Arg589 contacts three highly conserved charged residues—Glu^{187}, Asp^{602} and Asp^{131}. A possible explanation is, thus, that Trp589 would destabilize the tertiary structure of SDHA by eliminating polar contacts and by introducing a bulky side chain into the constrained space formed by the side chains surrounding Arg589.

Using immunohistochemistry analyses in the patient’s tumor, we showed that consequences of SDHA mutation are similar to those seen in other SDHx mutants but distinct from RET- or NF1-related tumors. All the SDHx-related tumors including the SDHA mutant reported here are deficient in SDHB expression, show HIF-1α protein stabilization and display high blood vessel density. By the same token, transcriptome analysis of gene expression in oxidative phosphorylation and glycolysis indicated a pattern in the SDHA-related tumor following that of other SDHx-related tumors. Consistent with the pheochromocytoma microarray study previously published by Dahia et al. (20), the microarray analysis we performed with HIF target genes showed two different clusters, one characteristic of the NF1- and RET-related pheochromocytomas and the second of SDHx- and VHL-related tumors. These data revealed that the SDHA patient’s tumor was classifiable in the VHL- and SDHx-paraganglioma/pheochromocytoma group and that like other SDHx tumors, it also displays an activated hypoxic pathway.

The absence of any SDHA-associated paraganglioma or pheochromocytoma was previously thought to be the consequence of the possible existence of two different SDHA genes, ‘type I Fp’ and ‘type II Fp’, with tissue-specific expression (12). Although ‘type I Fp’ SDHA was located on chromosome 5 (5p15) (6), the location of the putative ‘type II Fp’ intronless gene was never identified. Our data are not in favor of the existence of an intronless gene encoding ‘type II Fp’ since we identified the nucleotides specific of

Figure 4. Expression of SDHA, SDHB and COX-IV in SDHA-mutated paraganglioma compared with other inherited paraganglioma/pheochromocytoma. SDHA-positive immunostaining is observed in the chromaffin cells of SDHB- and RET-related tumors. In the patient’s tumor, it is detected solely in blood vessels and not in tumor cells. SDHB protein expression is lost in both SDHA- and SDHB-mutated tumors, whereas it is still present in RET-related pheochromocytomas. Subunit COX-IV of mitochondrial cytochrome c oxidase is expressed at comparable levels in all tumors. Calibration bar: 100 μm.
‘type II Fp’ even when intronic primers were used. They do not support either the tissue specificity of two isoforms since we observed the three possible conditions (‘only I Fp’, ‘only II Fp’ or ‘I + II Fp’) in pheochromocytoma of different individuals. Additionally, in agreement with another report (14), the two sequences displayed strong linkage disequilibrium in our control population. On the basis of these findings, we conclude that the ‘type I Fp’ and ‘type II Fp’ reported by Tomitsuka et al. (12) are most likely the result of frequent naturally occurring SDHA polymorphisms.

An alternative explanation was proposed by Guzy et al. to understand why SDHB, SDHC and SDHD mutations are associated with a tumor phenotype, whereas SDHA mutations are not (21). These authors suggested that mutations in SDHB, SDHC and SDHD would be expected to cause both a reactive oxygen species (ROS) (20) and succinate-mediated HIF-α stabilization leading to tumor progression. In contrast, SDHA mutations would act by increasing succinate alone. Using pharmacological inhibition or RNA interference in vitro, they did not detect any increase in normoxic ROS production,

Figure 5. Pseudohypoxia and angiogenesis in SDHA-mutated tumor compared with other inherited paraganglioma/pheochromocytoma. (A) HIF1-α nuclear immunostaining is detected in tumors with mutations in either SDHA- or SDHB- but not in RET-related pheochromcytomas. Calibration bar: 100 μm. CD34 immunohistochemistry was performed to evaluate angiogenesis in the SDHA-mutated paraganglioma and compared with other inherited paraganglioma/pheochromocytoma. Histogreen was used as a chromogen for detection (blue labeling). Quantification of vascular density reveals that the SDHA-mutated paraganglioma is highly vascularized, as are other SDHx-related tumors. (B) Unsupervised hierarchical clustering analysis of 69 samples according to the expression of 54 HIF-1 and/or HIF-2 targets (122 probes). Expression profiles are shown as a heat map indicating high (red) and low (green) expression according to a log2-transformed scale. The higher bipartition allows pseudo-hypoxic VHL- (white) and SDHx- (gray) to be distinguished from RET and NF1 (black) tumors. The SDHA paraganglioma (in yellow indicated by an asterisk) is classified with SDHx tumors.
HIF-α stabilization or tumor progression in cells lacking SDHA as opposed to those lacking SDHB. Our data, however, show that the SDHA mutation does lead to HIF stabilization and the subsequent activation of the hypoxic pathway. This is consistent with Selak et al. (22) results, who demonstrated that siRNA inhibition of SDH expression stabilizes HIF-1α by a mechanism independent of ROS production and only mediated by succinate accumulation.

At present, the most credible explanation for the rarity of SDH-related tumors is the relatively low frequency of 5p15 loss, the chromosomal region containing the SDHA locus, compared with the 1p36 (SDHB) and 11q23 (SDHD) loci that often undergo losses in tumor tissues. This was observed in our large series of 202 paraganglioma and pheochromocytoma samples analyzed by BAC array CGH and was previously reported by other groups (23–26). Interestingly, the rarity of SDHC-related tumors (27) may also be explained by the low frequency of LOH observed for the 1q21 chromosomal region containing SDHC locus.

This study adds SDHA to the list of genes coding for TCA cycle proteins involved in paraganglioma/pheochromocytoma tumorigenesis. SDHA mutations should be suspected in patients affected by paraganglioma or pheochromocytoma in the case of negative SDHA immunohistochemistry in the tumor tissue, when loss of SDH activity is found in tumor, despite negative SDHx genetic testing and/or when loss of 5p15 chromosome is found in tumor. Our study highlights the power of immunohistochemical and genomic techniques for the molecular characterization of paraganglioma/pheochromocytoma, approaches which can also provide valuable information for individually tailored genetic counseling.

MATERIALS AND METHODS

Case report

A 32-year-old woman developed pregnancy-induced hypertension at 30 weeks of gestation. No signs or symptoms of hyperadrenergic state were present during pregnancy. A few days after childbirth, hyperadrenergic symptoms appeared (dizziness, tachycardia and sweating) with hypertension (170/110 mmHg). High concentrations of urinary normetanephrine (12.12 μmol/24 h, normal range 0.5–2.40), urinary norepinephrine (4.287 nmol/24 h, normal range 70–500) and chromogranin A (944 mg/L, normal value <86.5) were measured. Urinary metanephrine, dopamine and plasmatic vanilmandelic acid levels were normal (Supplementary Material, Table S1). Magnetic resonance imaging disclosed a left adrenal mass (58 mm, suggestive of a pheochromocytoma or paraganglioma—iso-signal in T1-weighted images, high signal in fat-saturated T2-weighted images enhanced after gadolinium injection—(Supplementary Material, Fig. S2A). Computed tomography scan revealed a hypervascularized tumor (data not shown). Whole body meta-iodobenzylguanidine scintigraphy showed a single intense uptake in the left adrenal area (Supplementary Material, Fig. S2B) without any other localization. Following left adrenalectomy by laparoscopy, histology diagnosed a 47 mm extra-adrenal paraganglioma (Supplementary Material, Fig. S2C). Immediately after surgery, blood pressure and urinary normetanephrine returned to normal. There was no history of paraganglioma/pheochromocytoma known in her family or any syndromic lesion suggesting neurofibromatosis type 1, multiple endocrine neoplasia type 2 or von Hippel–Lindau disease.

Biological material

Patients signed a written informed consent for germline and somatic DNA analyses. Fresh tumor samples were collected during surgery and immediately frozen in liquid nitrogen. Germline DNAs were extracted from leukocytes according to standard protocols. Tumor DNAs and RNAs were extracted using AllPrep DNA/RNA Mini Kit (Qiagen). Total RNA was submitted to a DNAse I RNase-free treatment (Roche) and retrotranscribed using M-MLV Reverse Transcriptase (Invitrogen) and random hexamers.

A Caucasian reference population was used to obtain 370 control DNAs. Control RNAs were extracted from fibroblast cultures from healthy control individuals and from pheochromocytoma tumor tissue of patients collected by the COMETE network (1). Ethical approval for the study was obtained from the institutional review board (CPP Paris-Cochin, January 2007).

Mutation analysis

Mutation analysis for RET, VHL, SDHB, SDHC, SDHD and SDHAF2 genes was performed by direct sequencing. VHL, SDHB, SDHC and SDHD were also analyzed for the presence of large deletions as described previously (1,27).

SDHA cDNA was sequenced in five overlapping amplicons. To amplify each fragment by PCR, at least one primer was systematically designed overlapping two exons. The 15 exons and the intron–exon boundaries of SDHA in the germ-line and tumor DNAs were sequenced with primers specific for the SDHA gene on chromosome 5p15 but not for the pseudo-genes known to be present on chromosomes 5 and 3q29 (Supplementary Material, Table S2). In silico predictions were performed, based on sequence similarity, amino acid composition, protein structure and function (SIFT predictor, http://sift.jcvi.org/ and PolyPhen predictor, http://coot.embl.de/PolyPhen/ websites).

Respiratory chain activities

Cytochrome c oxidase (complex IV), succinate cytochrome c reductase (complex II +III) and quinol cytochrome c reductase (complex III) activities were measured spectrophoto metrically in pheochromocytoma or paraganglioma homogenates as described previously (28,29).

Microarray

Microarray analyses were performed as described previously (16). Tumor samples (20–30 mg) were powdered under liquid nitrogen. RNAs were extracted using RNeasy mini kit (Qiagen). Aliquots of the RNA were analyzed by electrophoresis on a Bioanlyser 2100 (Agilent Technologies) and quantified using Nano Drop ND-1000 (Labtech). Stringent criteria
for RNA quality were applied to rule out degradation, especially a 28S/18S ratio above 1.5. Microarray analyses were performed on 3 μg of total RNA for each sample and 10 μg cRNA per hybridization (GeneChip Fluidics Station 400; Affymetrix, Santa Clara, CA, USA). Total RNA were amplified and labeled following the manufacturer’s one-cycle target labeling protocol (http://www.affymetrix.com). The labeled cDNA were then hybridized to HG-U133 Plus 2.0 Affymetrix GeneChip arrays (Affymetrix). The chips were scanned with a GCOS 1.4.

BAC array CGH
Genomic DNA from paraganglioma or pheochromocytoma from 202 patients collected by the COMETE network was analyzed with IntegraChip (IntegraGen, Evry, France), a CGH microarray containing 4434 BACs. Raw log2-ratio feature values were filtered and remaining values were normalized using the lowess within-print tip group method (30). AWS smoothing technique was then applied to the normalized log2-ratio values [R package GLAD v1.8]. This yielded segments along the chromosome of homogeneous smoothed log2-ratios values. Thus, we assigned the smoothed CGH data into three different groups: gain, no change or loss.

Haplotype and linkage disequilibrium analysis
Linkage disequilibrium, carried out with THESIAS software (www.genecanvas.org), was deduced from the estimated haplotype frequencies, and its extent was expressed in terms of D’, this being the ratio of the non-standardized coefficient to its maximal and minimal values.

Studies on yeast SDH1 mutants
The SDH1 gene of S. cerevisiae is the homologue of human SDHA. An SDH1::HIS3 null allele was constructed by replacing the coding sequence between the two BglII sites internal to SDH1 with HIS3. The respiratory-deficient S. cerevisiae mutant aW303ΔSDH1 with the deleted SDH1 was obtained by the one-step gene replacement method (31).

Yeast SDH1 plus flanking 5’ and 3’ sequences was cloned in the integrative plasmid YIp352 (32). This construct was used to change the CGT codon 582 of SDH1 to TGG with the QuickChange II Site-Directed Mutagenesis Kit® (Stratagene). Complete sequences were obtained of WT SDH1 in plasmid pG52/ST19 and of the mutant SDH1 in plasmid pG52/ST19. The two sequences were identical to those reported in the database (http://www.yeastgenome.org/) except for the TGG codon of the gene in pG52/ST19. The WT and mutant SDH1 were integrated at the chromosomal URA3 locus of the null mutant aW303ΔSDH1.

Immunohistochemistry
Paraffin blocks were cut and 6 μm thick sections were mounted on Superfrost plus slides. Immunohistochemistry was performed as described using antibodies as follows (33): anti-SDHB (HPA002868, Sigma-Aldrich, 1/500), anti-SDHA (abcam, ab14715, 1/1000), anti-COX-IV (abcam, ab33985, 1/1000), anti-HIF1α (H1alpha67, abcam, 1/500) and anti-CD34 (Clone QBEND 10, Immunotech, 1/100). The protocol involved a biotinylated secondary antibody (Vector Laboratories), an avidin–biotin–peroxidase complex (Vectorstain ABC Elite; Vector Laboratories) and Histogreen (Abcys) as a chromogen.

Quantification of vascular density
Vascular density was measured on sections after CD34 immunostaining as described previously (16). Total blood vessels were counted in eight randomly chosen fields of 0.65 mm².

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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