Mutations in the SDHB Gene Are Associated with Extra-adrenal and/or Malignant Phaeochromocytomas

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Abstract

Germ-line mutations in the genes encoding succinate dehydrogenase complex subunits B (SDHB) and D (SDHD) have been reported in familial paragangliomas and apparently sporadic phaeochromocytomas (ASP), but the genotype-phenotype relationships of these mutations are unknown. Eighty-four patients (all but 2 followed up for 8.8 ± 5.7 years) with ASP (57 with adrenal tumors, 27 with extra-adrenal, multiple, malignant, or recurrent tumors) were screened for the major susceptibility genes for phaeochromocytoma (RET, VHL, SDHD, and SDHB). Thirty-three tumors were available for molecular analysis, enzyme assays, and immunohistochemistry. No (0%) RET and 2 (2.4%) VHL mutations were detected. Only two coding single nucleotide polymorphisms in the SDHD gene (G12S and H50R) were found in 6 patients (7%). Conversely, six deleterious mutations in the SDHB gene were identified in 8 patients (9.5%). Ectopic site and recurrence or malignancy were strongly associated with SDHB mutations (7 of 8, 87%, versus 20 of 76, 26%; P = 0.001).

Somatic DNA analysis indicated a loss of heterozygosity at chromosome 1p36 (SDHD locus) in 16 of 33 cases (48%). A loss of heterozygosity at the SDHB locus was found in all tumors with SDHD mutation, and assays of respiratory chain enzymes showed a complete loss of complex II catalytic activity. The vascular architecture of tumors with SDHB mutations displayed features typical of malignancy. These data strongly suggest that SDHB gene is a tumor suppressor gene and that the identification of germ-line mutations in SDHB gene in patients with ASPs should be considered as a high-risk factor for malignancy or recurrence.

Introduction

Phaeochromocytomas are catecholamine-secreting tumors derived from neural-crest cells. These tumors generally arise from the adrenal medulla but may also develop from extra-adrenal tissues. They are usually benign, but new tumors or true recurrences, either benign or malignant, are observed in 10–20% of cases (1). Young age at onset, multiple or extra-adrenal locations, and a family history of disease are associated with the risk of new tumors. A large size (>50 mm) of the tumor and low ratio of plasma adrenaline to adrenaline plus noradrenaline concentrations have been associated with true recurrences or malignancy (2, 3).

Several familial diseases, such as VHL, disease, multiple endocrine neoplasia type 2, and NF1, predispose individuals to phaeochromocytoma (4, 5). However, the genes responsible for these three diseases account for only a small proportion of the genetic predisposition to isolated ASP (6).

Two new susceptibility genes were recently identified, SDHD and SDHB, encoding subunits D and B of the mitochondrial SDH, respectively (7, 8). The mitochondrial SDH complex catalyzes the oxidation of succinate to fumarate in the Krebs’ cycle and feeds electrons to the respiratory chain ubiquinone pool. Mutations in these two genes have been reported in families with hereditary paraganglioma. SDHD mutations are mainly associated with HNP s, whereas SDHB mutations are reported in HNP, familial phaeochromocytomas, and ASP (9).

Neumann et al. (10) recently tested 271 patients with ASP and identified 23 patients with a germ-line mutation in the SDHD (n = 11, 4%) or SDHB (n = 12, 4.5%) genes.

Inactivating germ-line mutations in SDHD or SDHB genes may be associated with a somatic LOH, with the complete loss of SDH activity and the activation of the hypoxic/angiogenic pathway in the tumoral tissues (11, 12) and with a malignant phenotype (12). To assess the phenotypic consequences of such mutations, we analyzed a series of 84 patients with ASP, including 27 patients with extra-adrenal, malignant, and/or recurrent phaeochromocytomas. In addition to genetic analysis, we searched for somatic LOH, assayed SDH activity, and analyzed the vascular architecture phenotype, which has been shown to be strongly associated with malignancy (13).

Materials and Methods

Patients. The 84 patients (36 men) with ASP included in this study were part of a French cohort of patients with phaeochromocytomas recruited in the COMETE network (3). We excluded patients with a personal or family history of HNP, MEN2A and 2B, VHL disease, or NF1. Patients’ initial work-up included a search for neurofibromas and café-au-lait pigmented spots, for retinal hemangioblastomas at fundoscopic examination, for renal or pancreatic tumors at computed tomography scan, and the determination of plasma thyrocalcitonin concentration before and after pentagastrin infusion. Patients with phenotypic clues for MEN2, VHL, or NF1 were also excluded. Malignancy was defined as the presence of lymph node or distant metastases at the initial intervention. Extra-adrenal disease was defined as the presence of the firstly discovered tumor located at an extra-adrenal site. Recurrence was defined as the reappearance of the disease or the occurrence of metastases during follow-up. Eighty-two of the 84 patients were followed up for an average duration of 8.8 ± 5.7 years (range: 0.4–32).

Tissues and Blood Samples. Written informed consent for germ-line and somatic DNA analysis was obtained from each subject, and the study was approved by an institutional review board (CCPRPB Paris-Cochin, July 1996). Peripheral DNA was obtained from venous blood samples. Among the 84 patients, 33 fresh phaeochromocytoma samples were obtained during surgery and immediately frozen in liquid nitrogen. Somatic (from phaeochromocytoma) and constitutive (from leukocytes) DNA was extracted according to standard protocols.

Genetic Testing of Germ-line DNA. The four exons of the SDHD gene, the eight exons of the SDHB gene, the three exons of the VHL gene, and six
exons (exons 10, 11, 13, 14, 15, and 16) of the RET gene were amplified and directly sequenced as described previously (11, 12, 14, 15). Search for LOH at 11q23 (PGL1) and 1p36 (PGL4). LOH analysis was performed for the 33 tumors and germline DNA samples, using two flanking microsatellite markers at each locus: D11S501 and D11S509 for PGL1 (11q23) corresponding to the SDHD gene and D15S507 and D15S199 for PGL4 (1p36), corresponding to the SDHB gene. For the seven available (in all except P038) inherited SDHB tumors, we used 11 fluorescent oligonucleotides (D1S243, D1S468, D1S2694, D1S244, D1S2667, D1S507, D1S199, D1S478, D1S2674, D1S2749, and D1S513) overlapping a 60-cM region between lp36.33 (telomere) and lp34.3. For these seven tumors, LOH was confirmed using tumor DNA extracted from the phaeochromocytoma homogenates used for enzymatic studies in a second run of PGL4 locus analysis. Germ-line and tumor DNA was amplified using the following amplification temperatures: 54°C for D15S243 and D15S2694 (with 5% DMSO), 55°C for D15S199 (with 5% DMSO), 56°C for D1S468-D1S244-D1S2667-D1S478, and 58°C for D1S513-D1S2674-D1S2749-D1S507. The amplification products were analyzed with an ABI 3700 instrument (Applied Biosystems) and the GeneScan Analysis 3.5 software (ABI Prism).

Enzyme Assays. Succinate cytochrome c reductase (complex II + III) and quinol cytochrome c reductase (complex III) activities were measured spectrophotometrically in phaeochromocytoma homogenates as described previously (16).

Immunohistochemistry. The paraffin blocks prepared for routine pathological examination of the tumors were obtained. They were cut into 7- µm thick sections, which were mounted on silane-treated slides. Immunohistochemistry was performed with an anti-CD34 antibody (Immunotech, Marseille, France) and an anti-CD3 antibody (DAKO, Trappes, France) at dilutions of 1:100 and 1:1000, respectively. The protocol used has been described elsewhere (17) and included a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA), an avidin-biotin-peroxidase complex (Vectastain ABC Elite; Vector Laboratories), and diaminobenzidine as a chromogen for the Peroxidase activity.

Statistical Analysis. The characteristics of the patients are expressed as mean ± 1 SD for continuous variable and counts and percentages for discrete variables. Phenotypic differences in quantitative traits were assessed, according to genotype, by Student’s t test or ANOVA. Differences in distributions of qualitative traits according to genotype were assessed by standard χ² analysis and Fisher’s exact test. Calculations were carried out with Statview version 5.0 (Abacus Concepts).

RESULTS

1. Characteristics of the Patients. Fifty-seven patients (68%) had a single benign adrenal phaeochromocytoma without subsequent recurrence. Six patients (7%) had extra-adrenal phaeochromocytoma and/or multiple locations at initial operation; five were located at the Zuckerkandl body and one in the urinary bladder. In one case (subject P188), both a tumor at the Zuckerkandl body and at the right adrenal gland were detected at the initial work up. Twenty-one patients (25%) had a malignant form with metastases documented at first intervention (7 patients, including 3 patients with an adrenal and 4 patients with an extra-adrenal primary tumor site) or during follow-up (14 patients, including 9 patients with an adrenal and 5 patients with an extra-adrenal primary tumor site). As expected, malignant tumors were larger (P < 0.0001) and heavier (P < 0.0001) than benign ones and accompanied by a higher levels of urinary metanephrine secretion (P = 0.003).

2. Germ-line DNA Analysis. Sixteen (19%) of the 84 patients had germlinal amino acid change in VHL, SDHD, or SDHB gene, but none (0%) had mutations in the RET proto-oncogene. Two (2.4%) had mutations in the VHL gene, six (7.1%) had coding polymorphisms in the SDHD gene, and eight (9.5%) had deleterious mutations in the SDHB gene (Table 1).

The two germ-line mutations in the VHL gene were missense mutations, one new (P25L) and one already described (Y156C).

The G12S amino acid change in the SDHD gene was identified in two patients (2.4%), one with a recurrent malignant phaeochromocytoma and the other with a benign form (Table 2). This amino acid change was also found in 5 of 94 control subjects (5.3%), a frequency consistent with a nonfunctional polymorphism. We also detected the noncoding SNP S68S described previously in four patients (5%), a proportion similar to that observed in controls (4.2%). This SNP was in linkage disequilibrium with G12S in 2 patients and present in 2 others patients with benign adrenal tumors. The H50R amino acid change mutation was found in 4 patients (4.7%), a proportion not significantly different from that observed in controls (1%). It should be noted that the patient with a urinary bladder phaeochromocytoma also harbored a deleterious mutation in the SDHB gene.

Six germ-line mutations in SDHB were identified in 8 patients (Table 3 and Fig. 1). Three missense mutations (A43P, R46Q, and R46G) in exon 2 were detected in 4 patients, all with extra-adrenal or malignant forms of phaeochromocytoma. A43P was identified in a patient who first underwent surgery at the age of 29 years for an extra-adrenal phaeochromocytoma (#P106) in the Zuckerkandl body. Three years later, this patient developed a recurrence of the primary tumor and multiple metastases (lymph node, pulmonary, and bone).

Table 1 Baseline characteristics of the 84 patients with apparently sporadic phaeochromocytoma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Adrenal tumor</th>
<th>Extra-adrenal tumor</th>
<th>With metastases at first intervention</th>
<th>Recurrence or metastases at follow-up</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>57</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Age at first intervention, years</td>
<td>45.1 ± 13.5</td>
<td>43.3 ± 14.3</td>
<td>44.3 ± 13.5</td>
<td>39.4 ± 12.5</td>
<td>0.57</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>24/33</td>
<td>24</td>
<td>21</td>
<td>6/8</td>
<td>0.84</td>
</tr>
<tr>
<td>Symptom duration, years</td>
<td>21.3 ± 3.3</td>
<td>5.5 ± 9.1</td>
<td>2.1 ± 4.3</td>
<td>1.6 ± 1.5</td>
<td>0.16</td>
</tr>
<tr>
<td>Hypertension duration, years</td>
<td>4.8 ± 6.7</td>
<td>7.1 ± 7.2</td>
<td>1.3 ± 1.2</td>
<td>5.8 ± 10.3</td>
<td>0.45</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24.3 ± 5.5</td>
<td>23.1 ± 2.6</td>
<td>20.7 ± 3.2</td>
<td>21.3 ± 3.9</td>
<td>0.24</td>
</tr>
<tr>
<td>Tumor diameter, µm²</td>
<td>49.7 ± 21.8</td>
<td>52.5 ± 10.8</td>
<td>92.5 ± 36.7</td>
<td>90.4 ± 41.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tumor weight, g</td>
<td>63 ± 94</td>
<td>92 ± 90</td>
<td>385.7 ± 220.9</td>
<td>354.1 ± 402.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Urinary metanephrines, µmol/day</td>
<td>33 ± 32</td>
<td>31 ± 16</td>
<td>51 ± 28</td>
<td>76 ± 48</td>
<td>0.003</td>
</tr>
<tr>
<td>A/A × NA (%)*</td>
<td>12.8 ± 15.6</td>
<td>0.7 ± 1.0</td>
<td>1.0 ± 1.5</td>
<td>8.0 ± 7.4</td>
<td>0.10</td>
</tr>
<tr>
<td>Mean follow-up, months</td>
<td>91 ± 51</td>
<td>65 ± 42</td>
<td>114 ± 75</td>
<td>175 ± 91</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Number followed up</td>
<td>57</td>
<td>4</td>
<td>7</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

* A: adrenaline; NA, noradrenaline.

* Mutations detected correspond to the G12S and H50R polymorphisms.

* Patient also had a SDHB mutation.
The R46Q mutation was found in a 54-year-old woman with a large, highly vascularized adrenal tumor, which extended into the heart with multiple pulmonary metastases, a case reported previously (12). The same mutation was identified in a 28-year-old patient (#P188) with a right adrenal tumor (65 mm) that was difficult to treat by surgery because of extensive vascular connections with the aorta and a 50-mm tumor of the Zuckerkandl body. The R46G mutation was found in a 31-year-old woman with an apparently benign left adrenal phaeochromocytoma (#P195). The surgeon described a highly abnormal vascularization of this tumor, with a large arterio-venous plexus on its posterior face. Two frameshift mutations were detected in exon 6 of the SDHB gene in 3 patients. The deletion of cytosine at position 725 (725 del c) was observed in a 37-year-old man (#P107) operated of a phaeochromocytoma located in the urinary bladder but not followed up. The deletion of two nucleotides at positions 754 and 755 (754–755 del tg) was detected in two unrelated patients with malignant phaeochromocytomas. The first, a 38-year-old man (#P038), underwent surgery for phaeochromocytoma in the Zuckerkandl body and presented, 1 year later, a lymph node and bone metastases. The 2nd patient, a 24-year-old woman (#P117), also underwent surgery for phaeochromocytoma in the Zuckerkandl body and developed lymph node and thoracic bone metastases 3 years later. The last mutation was a missense mutation in exon 7 of the SDHB gene (R230C) that was detected in a 32-year-old man (#P109), who underwent surgery for an...
apparently benign left adrenal phaeochromocytoma, but who went on to develop bone and lymph node metastases 5 years later.

Four of the 6 patients with an SDHB mutation and complete follow-up died from their disease. For the series as a whole, there was a very strong association between the ectopic site, recurrence, and/or malignancy and the presence of SDHB mutations (7 of 8, 87%, versus 20 of 76, 26%; $P = 0.001$). It was striking that although 5 of the 8 (62.5%) SDHB mutation-positive patients had extra-adrenal phaeochromocytomas, only 10 of the 76 (13%) SDHB mutation-negative patients had extra-adrenal tumors, this difference in distribution being highly significant ($P = 0.001$). Similarly, 5 of 6 (83%) SDHB mutation-positive patients, followed-up to present or death, had malignant phaeochromocytoma at the time of diagnosis or a malignant recurrence, whereas this was the case for only 16 of 76 (21%) SDHB mutation-negative patients; this difference was highly significant (83 versus 21%, $P = 0.004$).

### 3. Somatic DNA Analysis and Complex II Enzyme Assays.

LOH analysis was performed on the 33 available tumors (all of the SDHB-inherited tumors except P#038) and the corresponding germ-line DNA samples using two microsatellite markers at the SDHD (11q23) and SDHB (1p36) loci (Table 4). Chromosome 11q23 LOH was found in 7 (21%) cases, all corresponding to benign adrenal tumors. Chromosome 1p36 LOH was found in 16 (48%) cases, half of them corresponding to extra-adrenal or malignant tumors. Among these 8 cases, 7 had a germ-line SDHB mutation. The combination of an SDHB germ-line mutation and chromosome 1p36 LOH was associated in six of seven cases with extra-adrenal location or malignancy (86%), and all germ-line SDHB mutations were associated with a 1p LOH ($P = 0.002$).

We carried out further molecular and enzymatic analysis for seven frozen SDHB-mutation-positive tumors available (all except P#038). We used 11 fluorescent microsatellite markers and detected a complete LOH in the 1pter to 1p34.3 region in 100% of these tumors. Assays of SDH activity showed a complete and selective loss of mitochondrial complex II enzymatic activity in all these tumors (Table 5). It was not possible to distinguish benign adrenal (#P195) or extra-adrenal phaeochromocytomas that were not followed-up (#P107, #P188) from malignant tumors.

### 4. Vascular Architecture of the Tumors.

We further investigated the possible link between somatic SDHB gene inactivation and malignancy by studying blood vessels morphology in the paraffin-embedded samples of six of the eight SDHB-mutation-positive phaeochromocytomas. We recently showed that vascular morphology is strongly associated with malignancy in these tumors (13). We compared the immunohistochemical results obtained with anti-CD34 antibodies to detect endothelial cells (data not shown) and anti-α-actin antibodies for the identification of vascular smooth muscle cells with those obtained for three benign adrenal SDHB mutation-negative phaeochromocytomas. As expected, control tumors displayed a normal vascular pattern with small capillaries regularly distributed throughout the tumor sample (Fig. 2A–C). In contrast, the malignant SDHB mutation-positive tumors displayed the archetypal irregular motifs described previously (#P106, Fig. 2G; #P183, Fig. 2H; and #P038, Fig. 2F). The apparently benign adrenal (#P195, Fig. 2E) or extra-adrenal but not followed-up SDHB mutation-positive phaeochromocytomas (#P188, Fig. 2D and #P107, Fig. 2F) also displayed an irregular architecture. Although less stereotypical than for #P106, #P183, and #P038, the change in vascular morphology observed (arcs, networks, and parallel structures) was similar to that observed in invasive malignant tumors.

### DISCUSSION

The frequency of malignancy has been estimated at 13–26% for phaeochromocytomas as a whole (18). After the initial surgery, recurrence is highly unpredictable. For individuals, genetic analysis of a cancer is of value if it can result in the detection of a particular risk of recurrence or malignancy or if it can influence medical or surgical treatment. For families, genetic analysis is of potential value if it can detect asymptomatic subjects who will go on to develop symptoms, enabling them to benefit from earlier treatment. Our study indicates that SDHB gene mutations are a strong indicator of malignancy in ASPs.

One of the most striking features of our results is the higher frequency of mutations in SDHB (9.5%) than in other genes implicated in phaeochromocytoma. In particular, we found no mutation in

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**Table 4** Somatic LOH at chromosome 1p and 11q in 33 apparently sporadic phaeochromocytomas

<table>
<thead>
<tr>
<th>SDHB locus LOH chromosome 1p</th>
<th>SDHD locus LOH chromosome 11q</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>According to the phenotype</strong></td>
<td></td>
</tr>
<tr>
<td>Adrenal ($n = 21$)</td>
<td>$- $</td>
</tr>
<tr>
<td>Extra-adrenal + malignant ($n = 12$)</td>
<td>$+$</td>
</tr>
<tr>
<td><strong>According to the SDHB mutation</strong></td>
<td></td>
</tr>
<tr>
<td>SDHB negative ($n = 26$)</td>
<td>$17$</td>
</tr>
<tr>
<td>SDHB positive ($n = 7$)</td>
<td>$0$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$P$</th>
<th>$+$</th>
<th>$+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P$</td>
<td>$0.12$</td>
<td>$0.012$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$P$</th>
<th>$+$</th>
<th>$+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P$</td>
<td>$0.002$</td>
<td>$0.001$</td>
</tr>
</tbody>
</table>

$^a$ Differences in distributions were assessed by Fisher’s exact test.

**Table 5** Respiratory chain enzyme activity in phaeochromocytomas with or without SDHB mutations

<table>
<thead>
<tr>
<th>ID #</th>
<th>SDHB germ-line mutations</th>
<th>1p LOH</th>
<th>Succinate cytochrome c reductase (CII + CIII) (nmol/min/mg protein)</th>
<th>Quinol cytochrome c reductase (CIII) (QCCR/SCCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P106</td>
<td>A3J</td>
<td>+</td>
<td>&lt;0.5</td>
<td>142</td>
</tr>
<tr>
<td>P107</td>
<td>725 del c</td>
<td>+</td>
<td>1</td>
<td>39.5</td>
</tr>
<tr>
<td>P109</td>
<td>R230C</td>
<td>+</td>
<td>&lt;0.5</td>
<td>277</td>
</tr>
<tr>
<td>P117</td>
<td>754, 755 del tg</td>
<td>+</td>
<td>&lt;1</td>
<td>62.5</td>
</tr>
<tr>
<td>P183</td>
<td>R46Q</td>
<td>+</td>
<td>&lt;0.01</td>
<td>320</td>
</tr>
<tr>
<td>P188</td>
<td>R46Q</td>
<td>+</td>
<td>&lt;1</td>
<td>112.5</td>
</tr>
<tr>
<td>P195</td>
<td>R46G</td>
<td>+</td>
<td>1.5</td>
<td>160</td>
</tr>
<tr>
<td>SDHB mutation-positive tumors ($n = 7$)</td>
<td>+</td>
<td>&lt;0.8 ± 0.5</td>
<td>159 ± 65</td>
<td>&gt;203 ± 169</td>
</tr>
<tr>
<td>SDHB mutation-negative tumors ($n = 6$)</td>
<td>−</td>
<td>75 ± 30</td>
<td>189 ± 70</td>
<td>2.7 ± 0.6</td>
</tr>
</tbody>
</table>

$^a$ No SDHB or SDHD mutation was detected in control phaeochromocytoma group (SDHB mutation-negative tumors).
the RET gene and only 2 (2.4%) mutations in the VHL gene. These results contrast with those observed by the Freiburg-Warsaw-Columbus Phaeochromocytoma Study Group, who obtained mutation frequencies of 5% for RET and 11% for VHL in 271 individuals with ASP (10). A likely explanation is that we included only patients without a family history of VHL or NEM2, without retinal hemangioblastomas, and with normal thyrocalcitonin levels after pentagastrin testing. In Neumann’s report, 12 of the 13 patients positive for mutations of RET developed medullary thyroid carcinoma during the follow-up period (10). In the same study, 10 of the 30 carriers of VHL mutations presented associated features of VHL disease during follow-up. Finally, the low frequency of RET and VHL mutations in our patients with ASP is also consistent with previous reports for these diseases with the early onset of thyroid lesions almost always preceding phaeochromocytoma in MEN2A (19) and extra-adrenal lesions (mainly retinal hemangioblastomas, renal-cell carcinoma) in most VHL cases (except for the type 2C of the disease; Ref. 20).

The other surprising finding of our study was the difference in mutation frequency between the SDHB and SDHD genes. The proportion of SDHB genetic variations was high, but only two coding polymorphisms (G12S and H50R) and one silent (S68S) polymorphisms were identified in the SDHD gene. The possible functionality of the G12S variant has been subjected to some debate because this variant has been found in affected subjects (21–23) but also in phenotypically normal subjects (24). Our results confirm that this mutation is probably a polymorphism because it was observed in similar proportions in subjects with ASP (2 of 84) and in a set of controls (5 of 94, \( P = 0.33 \)). Similar debates have concerned the potential functionality of the H50R variant (22, 23), the original amino acid being conserved in the mouse SDHB gene. In our study, the marginal difference in the frequency of the H50R mutation in ASP (4 of 84) and control (1 of 94, \( P = 0.15 \)) subjects leaves some room for speculation, although one of the four cases of ASP was more likely to be accounted for by one mutation in SDHB gene. We thus analyzed SDH activity in one phaeochromocytoma with a germ-line H50R mutation but without LOH at the SDHD locus (#P168). Succinate cytochrome c reductase (123 nmol/min/mg protein) and quinol cytochrome c reductase (234 nmol/min/mg protein) activities were normal, providing additional evidence that this nucleotide change results in the production of a functional protein. On the whole, the absence of deleterious mutations in the SDHD gene in our cohort contrasts with the frequency of such mutations (4%) reported by Neumann et al. (10). This discrepancy may be partly accounted for by selection bias attributable to the absence of glomus tumors during follow-up and the expression of SDHD mutations principally as head and neck paragangliomas (25). Our results are more in accordance with those obtained by Benn et al. (26) who did not find any SDHD but six SDHB mutations in 7 patients with familial or ASPs.

Our most important finding is that the presence of germ-line mutations in the SDHB gene, but not in the SDHD gene, are strongly associated with extra-adrenal phaeochromocytomas and confer a high risk of recurrence or malignancy. In the presence of an SDHB mutation, the odds ratio for an extra-adrenal tumor as a primary site is very high, 19.8 (95% confidence interval 2.9–213). If we consider only the 82 patients with a complete follow-up, the odds ratio for recurrence or malignancy was 19 (95% confidence interval 1.9–910). These odds ratios must clearly be interpreted with caution because of the small number of SDHB mutation-positive subjects but are high enough to...
warrant prospective evaluation of genetic analysis of this locus as a provisional test for ASPs. This higher risk of malignancy and of tumor formation at an extra-adrenal site conferred by SDHB mutations is consistent with several previous observations. Neumann et al. (10) provided no information concerning malignancy for their 12 patients with SDHB mutations but stated that 50% of these patients presented extra-adrenal disease. A missense mutation (R242H) in exon 7 of the SDHB gene was recently identified in a proband with initial metastatic periadrenal and periortic paragangliomas, followed by bone metastasis 29 years later (27). Several abdominal paragangliomas were subsequently discovered in his 27-year-old son, who carried the same mutation. The strengths of our study are the longevity of our cohort (the 1st patient to undergo surgery did so in 1970), the systematic follow-up (8.8 +/- 5.7 years) of all but 2 patients, and the high frequency of recurrent and malignant tumors (25%). Five patients with SDHB mutations clearly displayed a malignant phenotype. Two patients, one with two secreting tumors (adrenal and Zuckerkandl body) and the other with a phaeochromocytoma in the urinary bladder, were lost to follow-up. The only SDHB mutation-positive patient with an apparently benign phaeochromocytoma has been followed-up for only 18 months. We assessed the probability of malignancy for these three tumors by studying their vascular architecture. Angiogenesis is indeed a critical step in tumor growth and metastatic invasion. On the basis of our previous observation of a vascular pattern characteristic of malignant phaeochromocytomas (13), we compared the vascular architecture of these three tumors with three benign adrenal SDHB mutation-negative and three clearly malignant SDHB mutation-positive phaeochromocytomas. The result was unequivocal, with an irregular pattern of vascularization and almost all of the characteristics of malignant status identified in the three tumors assessed.

What are the molecular causes of malignancy for the SDHB tumors? As reported previously for one patient with an SDHD mutation (11) and for one with an SDHB mutation (12), all germ-line SDHB mutations were associated with somatic LOH at the SDHB locus from 1pter to 1p34.3 and with complete somatic loss of SDH activity. After SDH inactivation, the interruption of the Krebs' cycle and its consequences for the induction of angiogenic genes, such as EPAS-1 and HIF-1α (11), and the generation of superoxides by the respiratory chain may all be involved in tumor formation (28). Germ-line mutations in FH, which encodes fumarate hydratase, were recently reported in a dominantly inherited syndrome associating ureteral fibroids, skin leiomyomata, and papillary renal cell cancer (29). All these recent findings highlight the unexpected role of mitochondrial proteins in tumor pathogenesis.

It is also possible that another gene included in the chromosome 1p region is lost in the SDHB mutation-positive malignant phaeochromocytomas. Our data suggest that the SDHB gene may be one of these tumor suppressor genes.

In conclusion, this study emphasizes the clinical usefulness of genetic testing of all patients with phaeochromocytoma because genetic defects are relatively frequent even in apparently sporadic tumors. The presence of a germ-line SDHB mutation should be considered as a high-risk factor for malignancy and recurrence and should lead to appropriate clinical management and strict follow-up.

APPENDIX

The members of the COMETE Network were as follows: Youssef Anouar, INSERM U-413, Rouen; Ivan Bachelot, Service d’Endocrinologie, Hôpital Michallon, Grenoble; Erno Baviera, Laboratoire d’Anatomo-pathologie, Hôpital Saint Joseph, Paris; Chérif Beldjord, INSERM U-129, Institut Cochin, Paris; Xavier Bertagna, Institut Cochin, Paris; Jérôme Bertherat, Institut Cochin, Paris; Patrick Bruneval, Laboratoire d’Anatomo-Pathologie, HEGP, Paris; Olivier Charbe, Service d’Endocrinologie, Hôpital Michallon, Grenoble; Edmond Chambaz, INSERM U-244, Grenoble; Eric Clauser, INSERM U-36, Collège de France, Paris; Pierre Corvol, INSERM U-36, Collège de France, Paris; Catherine Delarue, INSERM U-413, Rouen; Jean-Marc Duclos, Service d’Urologie, Hôpital Saint Joseph, Paris; Jacques Epelbaum, INSERM U-159, Paris; Judith Favier, INSERM U-36, Collège de France, Paris; Jean-Marc Kuhn, Groupe Hospitalo-Universitaire de Recherche en Hormonologie, Rouen; Yves Le Bouc, Laboratoire d’Explorations Fonctionnelles Endocrinienes, Hôpital Armand Trousseau, Paris; Xavier Jeunemaitre, Département de Génétique, HEGP, Paris; Christine Gicquel, Laboratoire d’Explorations Fonctionnelles Endocrinienes, Hôpital Armand Trousseau, Paris; Hervé Le Hir, CGM-CNRS, Gif-sur-Yvette; Hervé Lefebvre, INSERM U-413, Rouen; Albert Louvel, Laboratoire d’Anatomo-Pathologie, Hôpital Cochin, Paris; Pierre-François Plouin, Département d’Hypertension Artérielle, HEGP, Paris; Antoine Tabarin, Hôpital du Haut-Leveque, CHU de Bordeaux; Claude Thermes, Centre de Génétique Moléculaire, CNRS A 9061, Gif-sur-Yvette; Pierre Thomopoulos, Clinique des Maladies Endocrinienes et Métaboliques, Hôpital Cochin, Paris; Hubert Vuadob, INSERM U-413, Rouen.

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