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Mutations in the *SDHB* Gene Are Associated with Extra-adrenal and/or Malignant Phaeochromocytomas[1]

Anne-Paule Gimenez-Roqueplo,[2] Judith Favier, Pierre Rustin, Claudine Rieubland, Malvina Crespin, Valérie Nau, Philippe Khau Van Kien, Pierre Corvol, Pierre-François Plouin, and Xavier Jeunemaitre for the COMETE (COrtical and MEdullary Tumour) Network[3]

AP/HP, Hôpital Européen Georges Pompidou, Département de Génétique Moléculaire [A-P. G-R., C. R., M. C., V. N., P. K. V. K., X. J.]; INSERM U36, Collège de France [A-P. G-R., J. F., P. C., P-F. P., X. J.]; INSERM U393, Hôpital des Enfants Malades [P. R.]; and AP/HP, Hôpital Européen Georges Pompidou, Service d'Hypertension Artérielle [P. C., P-F. P.], Paris

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 (*SDHB* locus) in 16 of 33 cases (48%). A loss of heterozygosity at the *SDHB* locus was found in all tumors with *SDHB* 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[4] 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 HNPs, 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 (CCPPRB 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

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[2] To whom requests for reprints should be addressed, at Département de Génétique Moléculaire, Hôpital Européen Georges Pompidou, 20-40, rue Leblanc, 75015 Paris, France. Phone: 33 1 56 09 38 81; Fax: 33 1 56 09 38 84; E-mail: Anne-Paule.Gimenez@hop.egp.ap-hop-paris.fr.

[3] The members of the COMETE Network are listed in the Appendix.

[4] The abbreviations used are: VHL, von Hippel-Lindau; ASP, apparently sporadic phaeochromocytoma; SDH, succinate dehydrogenase; LOH, loss of heterozygosity; COMETE, Cortical and Medullary adrenal Tumors; MEN, multiple endocrine neoplasia; NF1, neurofibromatosis type 1; HNP, head and/or neck paraganglioma.

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 tumor and germ-line DNA samples, using two flanking microsatellite markers at each locus: D11S5011 and D11S5019 for *PGL1* (11q23) corresponding to the *SDHD* gene and D1S507 and D1S199 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 1p36.33 (telomere) and 1p34.3. 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 D1S243 and D1S2694 (with 5% DMSO), 55°C for D1S199 (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-α-actin 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 χ^2 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 P#188), 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 germinal 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 phaeochromytoma (#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 phaeochromocytomas*

Extra-adrenal tumors corresponding to phaeochromocytomas of the organ of Zuckerkandl (five patients) and of the urinary bladder (one patient).

Parameter	Adrenal tumor	Extra-adrenal tumor	With metastases at first intervention	Recurrence or metastases at follow-up	*P*
Number	57	6	7	14	
Age at first intervention, years	45.1 ± 13.5	43.3 ± 14.3	44.3 ± 13.5	39.4 ± 12.5	0.57
Sex (M/F)	24/33	2/4	4/3	6/8	0.84
Symptom duration, years	2.1 ± 3.3	5.5 ± 9.1	2.1 ± 1.5	1.6 ± 1.5	0.16
Hypertension duration, years	4.4 ± 6.7	7.1 ± 7.2	1.3 ± 1.2	5.8 ± 10.3	0.45
Body mass index, kg/m^2	24.3 ± 5.5	23.1 ± 2.6	20.7 ± 3.2	21.3 ± 3.9	0.24
Tumor diameter	49.7 ± 21.8	52.5 ± 10.8	92.5 ± 36.7	90.4 ± 41.1	<0.0001
Tumor weight	63 ± 94	92 ± 90	358.7 ± 220.9	354.1 ± 402.3	<0.0001
Urinary metanephrines, μmol/day	33 ± 32	31 ± 16	51 ± 28	76 ± 48	0.003
A/A + NA (%)[a]	12.8 ± 15.6	0.7 ± 1.0	1.0 ± 1.5	8.0 ± 7.4	0.10
Mean follow-up, months	91 ± 51	65 ± 42	114 ± 75	175 ± 91	<0.0001
Number followed up	57	4	7	14	
Germ-line mutations/polymorphisms					
RET	0	0	0	0	
VHL	2	0	0	0	
SDHD[b]	3	2	0	1[c]	
SDHB	1	2	2	3	

[a] A, adrenaline; NA, noradrenaline.
[b] Mutations detected correspond to the G12S and H50R polymorphisms.
[c] Patient also had a *SDHB* mutation.

Table 2 *Characteristics of the 6 patients with SDHD coding polymorphisms*

Polymorphism		Patients				Cases	Controls	
cDNA	Amino acid	Id #	Sex	Age (years)	Tumor characteristics	*n*/total	*n*/total	Statistical significance
G44A	G12S	P027	F	31	Adrenal benign	2/84	5/94	$P = 0.33$
		P058	F	31	Adrenal with metastases at follow-up			
A160G	H50R	P005	H	60	Adrenal benign	4/84	1/94	$P = 0.15$
		P023	H	52	Adrenal benign			
		P107[a]	H	37	Extra-adrenal (urinary bladder) with no follow-up			
		P168	F	48	Extra-adrenal (Zuckerkandl body)			

[a] Patient also had a frameshift mutation in the *SDHB* gene.

Table 3 *Genotype-phenotype relationships in patients with germ-line SDHB mutations*

Id #	SDHB mutation [nucleotide cDNA, codon (exon)]	Sex	Age (years)	Initial tumor site/size (mm)/weight (g) (ms)	Postoperative outcome
P038	754–755 del tg Frameshift (ex 6)	M	38	Zuckerkandl body/120/150	Retroperitoneal and bone metastases Died after three reinterventions, embolisations, and MIBG Total follow-up 87 months
P106	g261c A43P (ex 2)	M	29	Zuckerkandl body/50/ND[a]	Lymph node recurrences Died after reintervention and embolisation Total follow-up 110 months
P107	725 del c Frameshift (ex 6)	M	37	Urinary bladder/70/195	No follow-up after first intervention
P109	c822t R230C (ex 7)	M	32	Left adrenal gland/36/20	Bone and lymph node metastasis Follow-up 90 months
P117	754–755 del tg Frameshift (ex 6)	F	24	Zuckerkandl body/90/109	Lymph node and bone metastases Died after further intervention and vertebroplasty Total follow-up 72 months
P183	g271a R46Q (ex 2)	F	54	Right adrenal gland extended into the heart/140/590	Pulmonary metastasis Died from circulatory failure during intervention
P188	g271a R46Q (ex 2)	M	28	Zuckerkandl body/50/25 Right adrenal gland/65/42	No follow-up after first intervention
P195	c270g R46G (ex 2)	F	31	Left adrenal gland/60/60	No recurrence after 18 months of follow-up

[a] ND, not determined.

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

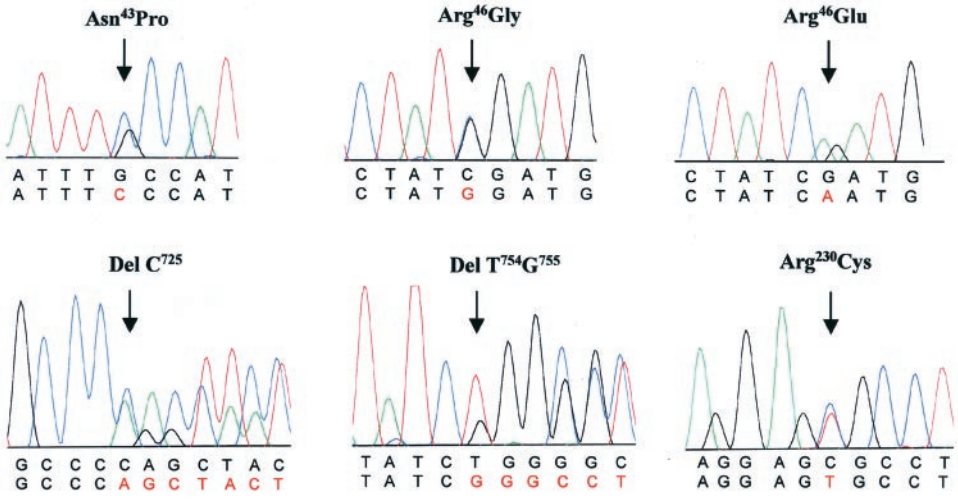

Fig. 1. Germ-line *SDHB* mutations. The sequence changes are indicated by *arrows*. The nucleotide change is mentioned in *red*.

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

	SDHB locus LOH chromosome 1p			*SDHD* locus LOH chromosome 11q		
	−	+	*P*	−	+	*P*
According to the phenotype						
Adrenal ($n = 21$)	13	8		14	7	
Extra-adrenal + malignant ($n = 12$)	4	8	$P = 0.12$	12	0	$P = 0.03$
According to the *SDHB* mutation						
SDHB negative ($n = 26$)	17	9		19	7	
SDHB positive ($n = 7$)	0	7	$P = 0.002$	6	1	$P = 0.49$

[a] Differences in distributions were assessed by Fisher's exact test.

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 germline 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 #P038). 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. 2*A–C*). In contrast, the malignant *SDHB* mutation-positive tumors displayed the archetypal irregular motifs described previously (#P106, Fig. 2*G*; #P183, Fig. 2*H*; and #P038, Fig. 2*I*). The apparently benign adrenal (#P195, Fig. 2*E*) or extra-adrenal but not followed-up *SDHB* mutation-positive phaeochromocytomas (#P188, Fig. 2*D* and #P107, Fig. 2*F*) 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

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

ID #	SDHB germ-line mutations	1p LOH	Succinate cytochrome *c* reductase (CII + CIII) (nmol/min/mg protein)	Quinol cytochrome *c* reductase (CIII) (nmol/min/mg protein)	QCCR/SCCR
P106	A43P	+	<0.5	142	>280
P107	725 del c	+	1	39.5	39.5
P109	R230C	+	<0.5	277	>500
P117	754, 755 del tg	+	<1	62.5	>62.5
P183	R46Q	+	<0.01	320	>320
P188	R46Q	+	<1	112.5	>112.5
P195	R46G	+	1.5	160	107
SDHB mutation-positive tumors ($n = 7$)		+	<0.8 ± 0.5	159 ± 05	>203 ± 169
SDHB mutation-negative tumors ($n = 6$)		−	75 ± 30	189 ± 70	2.7 ± 0.6

[a] No *SDHB* or *SDHD* mutation was detected in control phaeochromocytoma group (*SDHB* mutation-negative tumors).

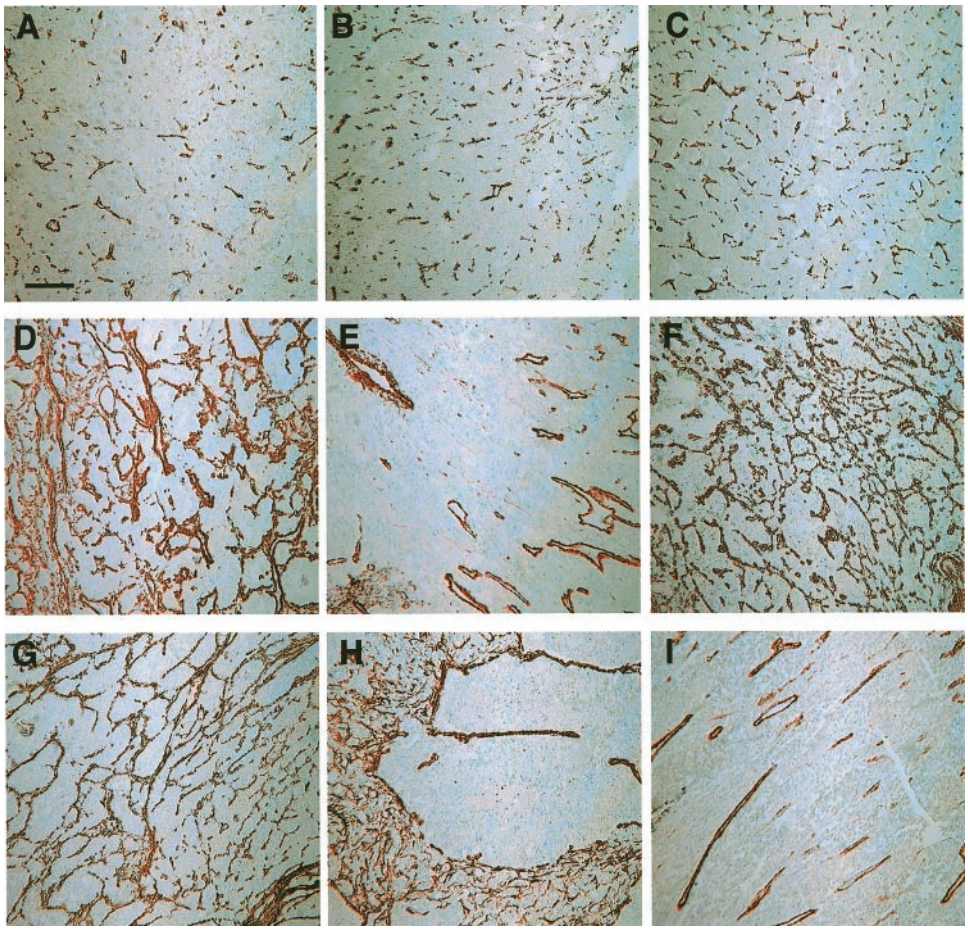

Fig. 2. Vascular architecture. The vascular morphology of three benign *SDHB* mutation-negative (*A–C*) and six *SDHB* mutation-positive [apparently benign (*D–F*) or malignant (*G–I*)] phaeochromocytomas was observed under a light microscope. The structure of blood vessels was studied by immunostaining the vascular smooth muscle cells with an anti-α-actin antibody. Note the ordered distribution of capillaries in the *SDHB* mutation-negative tissues and compare it with the irregular patterns observed in *SDHB* mutation-positive tumors, consisting of arcs and networks (*D* and *F–H*), avascular areas (*E* and *H*), or blood vessels running in parallel (*I*). Scale bar, 100 μm.

the *RET* gene and only 2 (2.4%) mutations in the *VHL* gene. These results contrast with those observed by the Freiburg-Warsaw-Columbus Pheochromocytoma 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 prognostic 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 periaortic 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 uterine 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 LOH is responsible for tumor formation. Several authors have suggested that chromosome 1p may carry tumor suppressor loci involved in phaeochromocytoma formation (30, 31). Two consensus LOH regions have been described in neuroblastoma, another neural crest-derived tumor: (*a*) one telomeric in 1p36 and (*b*) the other in 1p32–35. These LOH are usually screened as prognostic factors predictive of malignancy and used in treatment decisions (31). In sporadic and familial phaeochromocytomas, Benn *et al.* (30) have suggested three possible regions of common somatic loss: PC1; PC2; and PC3. The *SDHB* gene is exactly midway (1p36–13) between PC2 and PC3, which are 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 Chabre, 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-Jacques Feige, INSERM U-244, Grenoble; Jean-Marie Gasc, INSERM U-36, Collège de France, Paris; Anne-Paule Gimenez-Roqueplo, Département de Génétique, HEGP, Paris; Christine Gicquel, Laboratoire d'Explorations Fonctionnelles Endocriniennes, Hôpital Armand Trousseau, Paris; Xavier Jeunemaitre, Département de Génétique, HEGP, Paris; Jean-Marc Kuhn, Groupe Hospitalo-Universitaire de Recherche en Hormonologie, Rouen; Yves Le Bouc, Laboratoire d'Explorations Fonctionnelles Endocriniennes, 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, Hopital du Haut-Leveque, CHU de Bordeaux; Claude Thermes, Centre de Génétique Moléculaire, CNRS A 9061, Gif-sur-Yvette; Pierre Thomopoulos, Clinique des Maladies Endocriniennes et Métaboliques, Hôpital Cochin, Paris; Hubert Vaudry, INSERM U-413, Rouen.

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

Anne-Paule Gimenez-Roqueplo, Judith Favier, Pierre Rustin, et al.

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