The Succinate Dehydrogenase Genetic Testing in a Large Prospective Series of Patients with Paragangliomas

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Context: Germline mutations in *SDHx* genes cause hereditary paraganglioma.

Objective: The aim of the study was to assess the indications for succinate dehydrogenase (*SDH*) genetic testing in a prospective study.

Design: A total of 445 patients with head and neck and/or thoracic-abdominal or pelvic paragangliomas were recruited over 5 yr in 20 referral centers. In addition to classical direct sequencing of the *SDHB*, *SDHC*, and *SDHD* genes, two methods for detecting large genomic deletions or duplications were used, quantitative multiplex PCR of short fluorescent fragments (QMPSF) and multiplex ligation-dependent probe amplification (MLPA).

Results: A large variety of *SDH* germline mutations were found by direct sequencing in 220 patients and by QMPSF and MLPA in 22 patients (9.1%): 130 in *SDHD*, 96 in *SDHB*, and 16 in *SDHC*. Mutation carriers were younger and more frequently had multiple or malignant paraganglioma than patients without mutations. A head and neck paraganglioma was present in 97.7% of the *SDHD* and 87.5% of the *SDHC* mutation carriers, but in only 42.7% of the *SDHB* carriers. A thoracic-abdominal or pelvic location was present in 63.5% of the *SDHB*, 16.1% of the *SDHD*, and in 12.5% of the *SDHC* mutation carriers. Multiple paragangliomas were diagnosed in 66.9% of the *SDHD* mutation carriers. A malignant paraganglioma was documented in 37.5% of the *SDHB*, 3.1% of the *SDHD*, and none of the *SDHC* mutation carriers.

Conclusions: *SDH* genetic testing, including tests for large genomic deletions, is indicated in all patients with head and neck and/or thoracic-abdominal or pelvic paraganglioma and can be targeted according to clinical criteria. **(*J Clin Endocrinol Metab* 94: 2817–2827, 2009)**

Paragangliomas are highly vascularized tumors that can be benign or malignant and functional (with hypersecretion of catecholamines) or nonfunctional. They can be found in the head and neck and in the thoracic, abdominal (in the adrenal, catecholamine-secreting paragangliomas are called pheochromocytomas), and pelvic areas. The identification in 2000 and 2001 of the *SDHD*, *SDHB*, and *SDHC* genes encoding for three subunits of the succinate dehydrogenase enzyme has major implications for the genetics of paragangliomas and pheochromocytomas (1–3). Two large retrospective studies concerning patients affected by head and neck paragangliomas and/or thoracic, abdominal, or pelvic paragangliomas have been published and several mutations have been reported (4, 5). In 2005, our group reported that 10.2% of patients affected by pheochromocytoma or functional paraganglioma carry a germline mutation in the *SDHB* or *SDHD* genes (6). Consequently, recommendations

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Abbreviations: MLPA, Multiplex ligation-dependent probe amplification; QMPSF, quantitative multiplex PCR of short fluorescent fragments; SDH, succinate dehydrogenase; *VHL*, von Hippel Lindau gene.

for pheochromocytoma or functional paraganglioma genetic testing were proposed and applied by the international scientific community (7, 8).

In 2003, we set up a French paraganglioma network (PGL.NET) funded by the Groupement d'Intérêt Scientifique Institut des Maladies Rares and the Cortico et Médullosurrénale: les Tumeurs Endocrines (COMETE) network (9, 10). The objectives of the PGL.NET network include proposing genetic counseling to every patient affected by head and neck or thoracic-abdominal or pelvic paraganglioma and defining best practice for genetic testing of the affected patient. Thus, to determine appropriate indications and the recommendations for the practice of *SDH* genetic testing, we analyzed 445 patients with head and neck and/or thoracic-abdominal or pelvic paragangliomas recruited over 5 yr (2003–2008). We report here the mutation rates observed in the three genes known to cause paraganglioma and the genotype-phenotype relationships. We also show the value of using a technique, in addition to sequencing, to search for large genomic deletions or duplications that made up about 10% of the mutations that we detected.

Patients and Methods

Patients who presented only a single pheochromocytoma (unique adrenal catecholamine-secreting tumor) without another head and neck or thoracic-abdominal or pelvic paraganglioma and/or a family history of hereditary paraganglioma as well as patients suffering from a von Hippel Lindau disease were not included in the study. Patients affected by head and neck and/or thoracic-abdominal or pelvic paraganglioma (n = 445; 201 men and 244 women) were recruited in 20 French clinical centers. The procedures used for paraganglioma diagnosis were in accordance with institutional guidelines and have been described previously (4, 11, 12). In accordance with the French law, all patients attended an adapted interview with a physician and/or a geneticist to explain and propose the *SDH* genetic testing, and the study was approved by the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale des Pays de Loire (CCPPRB n°1 Angers). Written informed consent for DNA analysis was obtained from each patient.

Germline DNA was extracted from leukocytes according to standard protocols. All DNA were analyzed in the same genetic laboratory (at the Hôpital Européen Georges Pompidou in Paris). The coding portions and the exon-intron junctions of the three *SDHx* genes [succinate dehydrogenase subunit B, *SDHB* (RefSeq NM_003000.2), succinate dehydrogenase subunit C, *SDHC* (RefSeq NM_003001.3), and succinate dehydrogenase subunit D, *SDHD* (RefSeq NM_003002.1) genes (eight, six, and four exons, respectively)] were amplified by PCR and directly sequenced as previously described (6). Because direct sequencing is usually unable to detect large deletions or duplications of one or several exons, two additional screening methods were used. First, we adapted the quantitative multiplex PCR of short fluorescent fragments (QMPSF) method (13) to detect large deletions or duplications of the *SDHx* genes. The QMPSF method consists of a fluorescent multiplex PCR that enables simultaneous amplification of multiple short exonic fragments under quantitative conditions. All the *SDHB*, *SDHC*, and *SDHD* coding exons were screened by three different multiplex amplifications. Each multiplex PCR included five to nine sets of primers (Supplemental Table S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org) chosen with Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA). An additional fragment, from the hydroxymethylbilane synthase (*HMBS*) gene, was coamplified as a control in each PCR. A 5′ extension, consisting of a rare combination of 10 nucleotides preceding the exon-specific sequence, was added to primers as described previously (13). The forward primer of each pair was 5′ end-labeled with 6-FAM fluorochrome. Amplified DNA fragments were separated using an ABI PRISM 3730 DNA Analyzer sequencer (Applied Biosystems, Foster City, CA). Data were analyzed using the GeneMapper software version 4.0 (Applied Biosystems). Each QMPSF reaction was validated with positive and negative reference DNAs (11). Second, we used a commercially available kit, SALSA MLPA P226 (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. Multiplex ligation-dependent probe amplification (MLPA) is a semiquantitative method designed to detect deletions/duplications of one or more exons at the genomic level. The P226 kit includes nine specific probes for *SDHB*, six for *SDHC*, and five for *SDHD* to be used in one single reaction.

To assess the functionality of previously undescribed mutations (unknown variants), we sequenced 200 control chromosomes and used various strategies. For each missense unknown variant, we conducted *in silico* analyses (SIFT predictor, http://blocks.fhcrc.org/sift/SIFT.html; and PolyPhen predictor, http://coot.embl.de/PolyPhen/) based on sequence similarity analyses, the physical properties of amino acids, and the structure and function of human proteins. When frozen tumor tissues were available, we: 1) searched for exon skipping in cDNA for unknown variants located in splice sites (after extraction of tumor RNA and RT-PCR); 2) tested for loss of heterozygosity at the *SDH* locus using tumoral and peripheral DNA and microsatellite markers as described elsewhere (14, 15); or 3) measured succinate cytochrome c reductase (complex II + III) and quinol cytochrome c reductase (complex III) activities spectrophotometrically as described previously (14). When paraffin blocks were available, we tested for SDHB protein expression by immunohistochemistry as described previously (16), with a mouse monoclonal anti-SDHB antibody (Abcam, clone 21A11, 1/1000) and heat-mediated antigen retrieval in 10 mM citrate buffer (pH 6). For families with more than one member affected by paraganglioma, we analyzed the pedigree for the segregation of the mutation (Supplemental Table S2).

In addition, for all patients without *SDHx* mutation, the absence of von Hippel Lindau gene (*VHL*) mutation was also checked by sequencing and MPLA (kit SALSA MLPA P016; MRC Holland).

Statistical analyses were performed with Statview version 5.0 (SAS Institute Inc., Cary, NC) software. Differences between mutation carriers and non-mutation carriers, male and female, *SDHD* and *SDHB* mutation carriers, were assessed using the unpaired Student's *t* test for quan-

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titative variables and the χ^2 test or Fisher's exact test for qualitative variables. The possible interaction between gender and the presence of a mutation on several parameters was tested by logistic regression and by two-way ANOVA.

Results

General genetic data

The main clinical features of the 445 patients are reported in Table 1. A total of 330 patients (74.2%) had at least one or more head and neck paragangliomas, 139 (31.2%) had at least one or more thoracic-abdominal or pelvic paragangliomas, and 122 (27.4%) had multiple head and neck and/or thoracic-abdominal or pelvic paragangliomas. A malignant paraganglioma, defined by the presence of extraparaganglionic metastases (in lymph nodes, bone, liver, and lung), was diagnosed in 49 (11%) patients. A family history of paraganglioma was known for 103 patients (23.1%).

A germline mutation in *SDHD*, *SDHB*, or *SDHC* was identified in 242 patients (54.4%). As indicated in Table 1, they were on average 14 yr younger at the age of diagnosis than non-mutation carriers (36.2 ± 15.2 *vs.* 50.2 ± 15.3 yr), and 132 of them (54.5%) were younger than 36 yr at first diagnosis. Twenty-one patients developed tumors before the age of 18 yr (11 *SDHB*, 9 *SDHD*, 1 *SDHC* mutation carriers), four were children age 10 or younger, and 17 were between 11 and 17 yr of age. Mutations were found in relatively few (31 of 190 or 16.3%) of the patients with an apparently sporadic paraganglioma but in almost all patients with a positive family history (102 of 103 or 99%). Mutation carriers had multiple tumors and malignant disease more frequently than the non-mutation carriers (112 of 242 *vs.* 10 of 203, $P < 0.0001$; and 40 of 242 *vs.* 9 of 203, $P < 0.0001$, respectively). In our series, a higher proportion of women was observed in the non-mutation carriers (138 of 203 or 68%) than in the mutation carriers group (106 of 242 or 43.8%; $P < 0.0001$). In particular, there were 2.4 times more women than men among those with an apparently sporadic unique paraganglioma (112 *vs.* 47) or with a head and neck paraganglioma (112 *vs.* 36). This difference could reflect a bias in the detection of small tumors by self-observation in women.

The relation between the mutation status and the type of paraganglioma is indicated in Fig. 1A. A high rate of mutation was found in the case of multiple tumors (112 of 122 or 91.8%) but in approximately 40% of the patients with single paraganglioma whatever its location. All 24 patients affected by multiple

TABLE 1. Clinical features of all affected cases, the *SDH* mutation carriers and the *SDH* non-mutation carriers, at the time of genetic testing

Variables	All	Mutation carriers	Non-mutation carriers	Statistical effect of mutation, sex, interaction mutation-sex
No. of patients	445	242 [M, 136 (56.2%); F, 106 (43.8%)]	203 [M, 65 (32%); F, 138 (68%)]	–, $P <$ 0.0001, –
Age at first diagnosis of PGL, yr ± SD (range)	42.7 ± 16.7 (6–96)	36.2 ± 15.2 (6–96) [M, 37.2 ± 16.2; F, 34.9 ± 13.8]	50.2 ± 15.3 (11–83) [M, 47.7 ± 15.8; F, 51.3 ± 14.9]	$P <$ 0.0001, $P = 0.02$, $P = 0.05$
No. of patients with age at first diagnosis ≤35 yr	166	132 [M, 70 (53%); F, 62 (47%)]	34 [M, 15 (44.1%); F, 19 (55.9%)]	$P <$ 0.0001, $P = 0.35$, $P = 0.17$
No. of patients with PGL/PH family history	103	102 [M, 53 (52%); F, 49 (48%)]	1 [M, 0 (0%); F, 1 (100%)]	$P <$ 0.0001, –, –
No. of patients with multiple tumors (PGL >1)	122	112 [M, 66 (58.9%); F, 46 (41.1%)]	10 [M, 3 (30%); F, 7 (70%)]	$P <$ 0.0001, $P = 0.07$, $P = 0.48$
No. of patients with metastases	49	40 [M, 26 (65%); F, 14 (35%)]	9 [M, 5 (55.6%); F, 4 (44.4%)]	$P <$ 0.0001, $P = 0.87$, $P = 0.78$
No. of patients with an apparently sporadic PGL (single apparently benign tumor, age of onset >35 yr, and sporadic at presentation)	190	31 [M, 18 (58.1%); F, 13 (41.9%)]	159 [M, 47 (29.6%); F, 112 (70.4%)]	$P <$ 0.0001, $P = 0.002$, $P = 0.38$
No. of patients with head and neck PGL[a]	330	182 [M, 98 (53.8%); F, 84 (46.2%)]	148 [M, 36 (24.3%); F, 112 (75.7%)]	$P = 0.58$, $P <$ 0.001, $P = 0.03$
No. of patients with thoracic, abdominal, or pelvic PGL[a,b]	139	84 [M, 55 (66.5%); F, 29 (34.5%)]	55 [M, 29 (52.7%); F, 26 (47.3%)]	$P = 0.63$, $P = 0.13$, $P = 0.16$

The statistical comparisons between the repartition of mutation and non-mutation carriers and between males and females in each subgroup and the entire series of patients were tested independently by χ^2 analysis and logistic regression analysis. The statistical comparison for the age at first diagnosis according to the mutation status and gender was performed by a two-way ANOVA. *Dashes* indicate that the statistics could not be performed due to the presence of only one unfixed variable (sex in the numbers of mutation and non-mutation carriers), or of too small numbers (one non-mutation carrier in the patients with a family history). PGL, Paraganglioma; PH, pheochromocytoma; M, males; F, females.

[a] Twenty-four patients had both head and neck and thoracic, abdominal, or pelvic paragangliomas.

[b] Patients with a single (adrenal) pheochromocytoma, without another thoracic, abdominal or pelvic or head and neck paraganglioma and/or family history of hereditary paraganglioma were not included in the study.

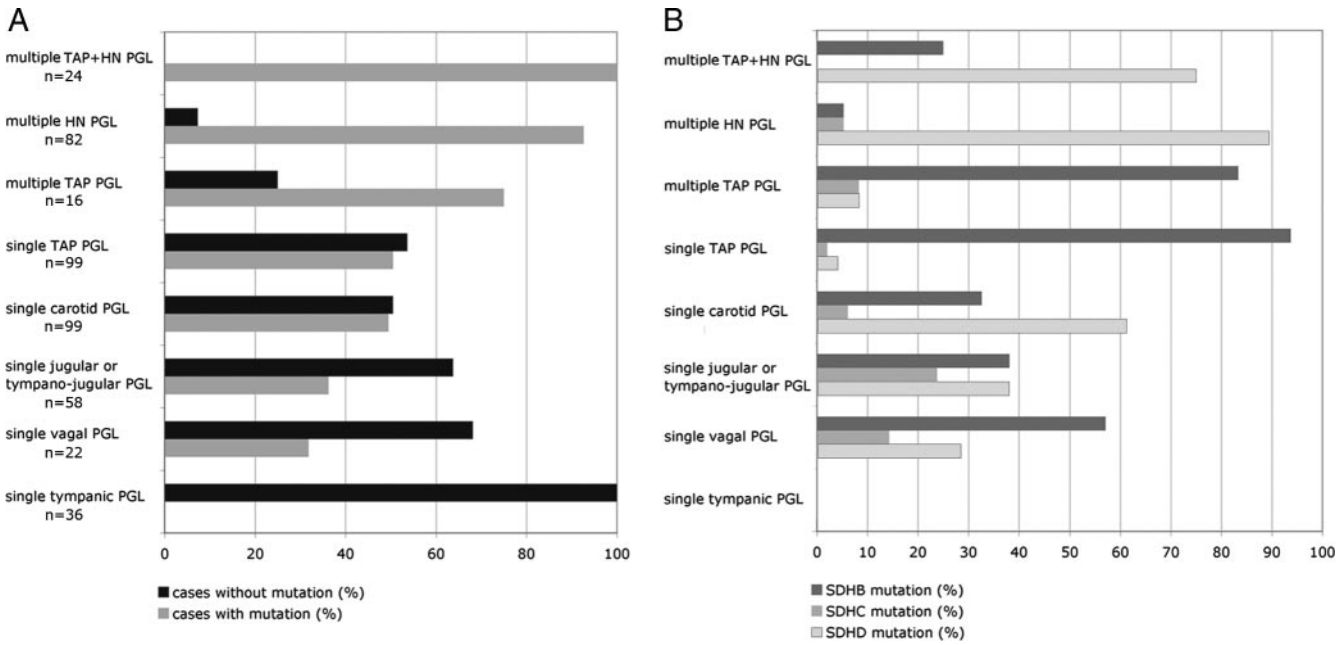

FIG. 1. A, Mutation status depending on the number of paragangliomas and their location. B, Repartition of *SDHB*, *SDHC*, and *SDHD* mutations depending on the number of paragangliomas and their location. Due to their small number, the nine patients with a single paraganglioma developed in the thyroid gland or in the larynx are not referred in the figure. TAP, Thoracic, abdominal, or pelvic; HN, head and neck; PGL, paraganglioma.

paragangliomas (thoracic-abdominal or pelvic and head and neck paragangliomas) had a mutation, compared with 92.7% (76 of 82) of those with multiple head and neck paragangliomas and 75% (12 of 16) of those with multiple thoracic-abdominal or pelvic paragangliomas. Half of the patients with a single carotid paraganglioma (49 of 99) but only 35% (28 of 80) of the patients with single jugular, tympano-jugular, or vagal paraganglioma were detected as positive, and none of those with single tympanic paraganglioma.

Characteristics of the SDH mutation carriers

The clinical characteristics of the *SDHB*, *SDHC*, and *SDHD* mutation carriers are given in Table 2 and Fig. 1B.

SDHD mutation carriers

A *SDHD* gene mutation was found in 130 subjects, the majority of them having a positive family history (59.2%) and multiple tumors (66.9%; median, 2.3; range, 1–8). All 21 patients with more than three paragangliomas had a *SDHD* mutation. Almost all *SDHD* mutation carriers had head and neck paragangliomas (127 of 130 or 97.7%) and mostly with a carotid location (109 of 127 or 85.8%). A head and neck paraganglioma (unique or multiple) is thus a strong predictor of a *SDHD* mutation. The presentation of the disease was benign in most of the cases, with only four of the 130 patients suffering from a malignant paraganglioma (3.1%). The mean age at diagnosis was 35.7 yr, the earliest and the latest diagnosis being made at 10 and

TABLE 2. Clinical characteristics of the *SDHB*, *SDHC*, and *SDHD* mutation carriers

Variables	*SDHB* mutation carriers	*SDHC* mutation carriers	*SDHD* mutation carriers	Effect of gene *P* value
n	96	16	130	
Sex (males/females)	52/44	9/7	75/55	0.87
Age at first diagnosis of PGL, yr ± SD (range)	36.7 ± 15.3 (6–77)	38.2 ± 13.3 (17–70)	35.7 ± 15.5 (10–96)	0.63
Patients with age at first diagnosis ≤35 yr, n (%)	48 (50)	6 (37.5)	78 (60)	0.12
Patients with family history, n (%)	21 (22)	4 (25)	77 (59.2)	<0.0001
Patients with multiple tumors, n (%)	20 (20.8)	5 (31.2)	87 (66.9)	<0.0001
Patients with metastases, n (%)	36 (37.5)	0	4 (3.1)	<0.0001
Patients with an apparently sporadic PGL (single apparently benign tumor, age of onset >35 yr, and sporadic at presentation), n (%)	19 (19.8)	5 (31.2)	7 (5.4)	0.0004
Patients with head and neck PGL, n (%)	41 (42.7)	14 (87.5)	127 (97.7)	<0.0001
Patients with thoracic, abdominal, or pelvic PGL, n (%)[a]	61 (63.5)	2 (12.5)	21 (16.1)	<0.0001

PGL, Paraganglioma.

[a] Patients with a single (adrenal) pheochromocytoma, without another thoracic, abdominal, or pelvic or head and neck paraganglioma and/or family history of hereditary paraganglioma were not included in the study.

96 yr of age, respectively. After 60 yr of age, only 15 patients (11.5%) had a thoracic-abdominal or pelvic paraganglioma.

SDHB mutation carriers

A *SDHB* gene mutation was detected in 96 subjects, with only a small proportion (20.8%) with multiple paragangliomas (median, 1.3; range, 1–3) and a positive family history (22%). Sixty-one (63.5%) of the *SDHB* mutation carriers had a thoracic-abdominal or pelvic paraganglioma, 45 of them being catecholamine-secreting (11 pheochromocytomas). As expected, paragangliomas were often malignant (37.5%). The mean age at diagnosis was comparable to the other mutated groups (36.7 yr). Two patients developed the first paraganglioma before 10 yr of age and nine (9.4%) before 15 yr. A total of 88 subjects (92%) had developed the disease by the age of 60 yr: 33 (34%) had a head and neck paraganglioma, and 52 (54.2%) had a thoracic-abdominal or pelvic paraganglioma.

SDHC mutation carriers

A *SDHC* gene mutation was found in 16 patients corresponding to 6.6% of all mutation carriers. Fourteen of them had a head and neck paraganglioma, and two had a functional thoracic paraganglioma. The youngest *SDHC* mutation carrier was 17 yr old at diagnosis and had a thoracic tumor.

SDHx mutation type

Among the mutation carriers, 220 had a splice-site or an intra-exonic mutation or a small deletion or a small insertion (90.9%). Twenty-two had a large deletion (9.1%). Ninety-eight different germline *SDH* mutations were identified (Table 3). Most of them (n = 63) were already described in the tricarboxylic acid Cycle Gene Mutation *Database* (17) but 35 new mutations are reported herein. *In silico* analysis predictions were performed for every new missense mutation: all predictions were consistent with a protein-damaging effect (Supplemental Table S2). Direct sequencing revealed 34 missense (23 on *SDHB*, four on *SDHC*, seven on *SDHD*), 26 frame-shift (12 on *SDHB*, one on *SDHC*, 13 on *SDHD*), 14 splice (seven on *SDHB*, one on *SDHC*, six on *SDHD*), 12 nonsense (three on *SDHB*, two on *SDHC*, seven on *SDHD*), and four in frame (one on *SDHB*, one on *SDHC*, two on *SDHD*) mutations. The QMPSF and MLPA methods were used in the 225 subjects with a negative *SDH* direct sequencing. Large deletions were found in each of the three genes and were identified by both methods (four involving *SDHB*, two *SDHC*, and two *SDHD*). Among them, two occurred at the first exon of *SDHB*, one of about 20 kb previously described by Cascon *et al.* (18), and a shorter one of around 2.5 kb in size (Supplemental Fig. S1). Twenty-eight single nucleotide polymorphisms were also detected (Supplemental Table S3). Nineteen of them are reported (in http://chromium.liacs.nl/lovd_sdh/ or www.ncbi.nlm.nih.gov/projects/SNP/). The other nine were also found in the control population or, in two cases, were associated with a clearly deleterious mutation.

Recommendations for SDH genetic testing

Our data demonstrate that all patients, except those with a single tympanic paraganglioma, should have a *SDH* genetic testing. The genetic testing should involve direct sequencing first; if negative, this should be followed by the search for large genomic deletions.

In our series, among the 224 patients with a unique head and neck paraganglioma at presentation, mutations were identified in all three *SDHx* genes (Table 4 and Fig. 1B). However, for cases with a family history of paraganglioma or when the age at diagnosis was 35 yr or less, most mutations were found in the *SDHD* (58.3 and 68.4%, respectively) and the *SDHB* (30.6 and 23.7%), and fewer were found in the *SDHC* gene (11.1 and 7.9%). For patients with head and neck paraganglioma, an age at diagnosis below 35 yr, and an apparently benign tumor with a sporadic presentation, a mutation was identified in only 16.7% of the cases (52% in *SDHB*, 28% in *SDHD*, and 20% in *SDHC*).

Among the 99 patients with a single thoracic-abdominal or pelvic paraganglioma, irrespective of the clinical presentation, 83 to 100% of all mutations detected were in the *SDHB* gene; only one *SDHC* mutation was found in this group.

For the 122 patients who had multiple tumors at presentation, most mutations were in the *SDHD* gene. However, in cases with malignant form, irrespective of the number of tumors and of their location, most mutations were in the *SDHB* gene (76.9 to 100% of mutations), and none were found in the *SDHC* gene.

Discussion

We reported herein a large study concerning genetic testing for patients with paraganglioma. The mutation yield was high with the identification of 242 *SDHD*, *SDHB*, or *SDHC* mutation carriers (54.4%) among the 445 patients analyzed. This higher prevalence of mutations than reported in previous studies (4, 5) is probably due in part to greater accuracy of the *SDH* genetic screening as a consequence of systematic testing for large genomic deletions; this identified a significant number of additional mutation carriers (9.1% of the total *SDHx* mutations). Also, our series of patients had relatively severe disease (37% of them had an early age of onset, 23% had a family history of paraganglioma or pheochromocytoma, 27.4% had multiple tumors, and 11% had malignant forms). However, mutations were also found in 16.3% of the 190 cases not suspected to carry (patients older than 35 yr at onset or having a single apparently benign paraganglioma and sporadic presentation). In addition, we have observed in our series an excess of women with a unique head and neck paraganglioma among the non-mutation carriers, suggesting that women detect more easily the tumors developed in head and neck and consult more rapidly than men in French clinical centers. This gender difference in early detection of cancer has already been reported (for review, see Ref. 19). Interestingly, we did not find a mutation in any patient with a single tympanic paraganglioma. Our findings indicate that all patients with paraganglioma, except those with a single tympanic paraganglioma, should benefit from genetic testing focused according to simple clinical data. Based on results in Table 4 and Fig. 1B, we propose an algorithm (Fig. 2) dedicated to geneticists and molecular genetic laboratories to organize and focus genetic testing according to simple clinical data at presentation. Applied to

TABLE 3. List of identified germline SDH mutations

Gene	Exon, intron	Mutation cDNA nucleotide change	Mutation amino acid change	Mutation type[a]	No. of cases	No. of families
SDHB	1	c.1-?_72+?del	DEL1	4	1	1
	1	c.1-16416_72 + 3877del	DEL1	4	5	4
	1	c.17_35del	p.Ala6GlyfsX65	1	2	1
	IVS1	c.72 + 1G>A[b]	c.72 + 1G>A	5	1	1
	2	c.88del	p.Gln30ArgfsX7	1	1	1
	2	c.127G>C	p.Ala43Pro	3	2	2
	2	c.136C>G	p.Arg46Gly	3	2	2
	2	c.136C>T	p.Arg46X	2	1	1
	2	c.137G>A	p.Arg46Gln	3	4	4
	2	c.166_170del	p.Pro56TyrfsX5	1	2	1
	2	c.167C>T	p.Pro56Leu	3	2	2
	IVS2	c.200 + 1G>A[b]	c.200 + 1G>A	5	1	1
	IVS2	c.201-1G>A[b]	c.201-1G>A	5	1	1
	3	c.201-?_286+?del[b]	DEL3	4	1	1
	3	c.206G>T[b]	p.Gly69Val	3	3	2
	3	c.260T>C	p.Leu87Ser	3	1	1
	3	c.268C>T	p.Arg90X	2	3	2
	IVS3	c.287-2A>G	c.287-2A>G	5	1	1
	4	c.314_316del[b]	p.Ile105del	6	3	2
	4	c.330_331del[b]	Leu111SerfsX7	1	1	1
	4	c.392del	p.Pro131HisfsX5	1	5	3
	IVS4	c.423 + 1G>A	c.423 + 1G>A	5	3	2
	IVS4	c.423 + 1G>C	c.423 + 1G>C	5	3	3
	5	c.470T>G[b]	p.Leu157Trp	3	1	1
	5	c.493del[b]	p.Glu165LysfsX10	1	1	1
	IVS5	c.540 + 2T>C[b]	c.540 + 2T>C	5	1	1
	6	c.546del[b]	p.Leu183SerfsX37	1	1	1
	6	c.565T>C[b]	p.Cys189Arg	3	2	2
	6	c.574T>C	p.Cys192Arg	3	1	1
	6	c.575G>A	p.Cys192Tyr	3	1	1
	6	c.587G>A	p.Cys196Tyr	3	2	2
	6	c.589C>T	p.Pro197Ser	3	1	1
	6	c.591del	p.Ser198AlafsX22	1	2	2
	6	c.617A>G[b]	p.Tyr206Cys	3	1	1
	6	c.620_621del	p.Leu207ArgfsX14	1	5	4
	7	c.649C>T	p.Arg217Cys	3	1	1
	7	c.654G>A[b]	p.Trp218X	2	1	1
	7	c.688C>T	p.Arg230Cys	3	2	2
	7	c.689G>A	p.Arg230His	3	5	4
	7	c.713del	p.Phe238SerfsX10	1	6	4
	7	c.718_719del[b]	Leu240IlefsX15	1	1	1
	7	c.718_721del	p.Leu240ThrfsX7	1	2	1
	7	c.724C>T	p.Arg242Cys	3	3	2
	7	c.725G>A	p.Arg242His	3	1	1
	7	c.745T>G[b]	p.Cys249Gly	3	1	1
	7	c.746G>A[b]	p.Cys249Tyr	3	1	1
	7	c.758G>A	p.Cys253Tyr	3	1	1
	7	c.761C>T	p.Pro254Leu	3	1	1
	7	c.763A>G[b]	p.Lys255Glu	3	1	1
	3 to 8	c.201-?_843 + ?del	DEL3–8	4	1	1
SDHC	1	c.1-?_20+?del[b]	DEL1	4	1	1
	1	c.1A>G	p.Met1?	1	1	1
	IVS1	c.21-2A>C[b]	c.21-2A>C	5	1	1
	2	c.21-?_77 + ?del[b]	DEL2	4	1	1
	2	c.43C>T	p.Arg15X	2	1	1
	4	c.214C>T	p.Arg72Cys	3	1	1
	4	c.215G>A[b]	p.Arg72His	3	5	3
	4	c.241G>C[b]	p.Gly81Arg	3	1	1
	5	c.253_255dup	p.Phe85dup	6	1	1
	5	c.397C>T	p.Arg133X	2	2	2
	6	c.473T>C	p.Leu158Pro	3	1	1

(*Continued*)

TABLE 3. (*Continued*)

Gene	Exon, intron	Mutation cDNA nucleotide change	Mutation amino acid change	Mutation type[a]	No. of cases	No. of families
SDHD	1	c.2T>C[b]	p.Met1?	1	7	5
	1	c.21del	p.Ser8ValfsX7	1	1	1
	2	c.64C>T	p.Arg22X	2	7	5
	2	c.112C>T	p.Arg38X	2	5	3
	2	c.129G>A	p.Trp43X	2	5	5
	2	c.139C>T[b]	p.Gln47X	2	1	1
	2	c.149dup	p.His50GlnfsX19	1	3	2
	IVS2	c.169 + 1G>T	c.169 + 1G>T	5	2	1
	IVS2	c.169 + 5G>A	c.169 + 5G>A	5	2	2
	IVS2	c.170-1G>T	c.170-1G>T	5	15	7
	3	c.191_192del	p.Leu64ProfsX4	1	1	1
	3	c.202dup	p.Ser68LysfsX46	1	1	1
	3	c.209G>C[b]	p.Arg70Thr	3	1	1
	3	c.209G>T	p.Arg70Met	3	1	1
	3	c.224del[b]	p.Leu75CysfsX11	1	1	1
	3	c.239T>G[b]	p.Leu80Arg	3	2	1
	3	c.242C>T	p.Pro81Leu	3	5	5
	3	c.242del[b]	p.Pro81ArgfsX5	1	1	1
	3	c.252T>G	p.Tyr84X	2	3	1
	3	c.254T>A	p.Leu85X	2	2	1
	3	c.260del[b]	p.Pro87LeufsX48	1	2	1
	3	c.275_286del[b]	p.Asp92_Leu95del	6	2	1
	3	c.305A>C[b]	p.His102Pro	3	1	1
	IVS3	c.314 + 1G>A	c.314 + 1G>A	5	3	1
	IVS3	c.315-2A>C[b]	c.315-2A>C	5	2	1
	IVS3	c.315-2A>G[b]	c.315-2A>G	5	1	1
	4	315-?_480+?del[b]	DEL4	4	7	3
	4	c.325C>T	p.Gln109X	2	3	1
	4	c.334_337del	p.Asp113MetfsX21	1	8	5
	4	c.341A>G	p.Tyr114Cys	3	3	2
	4	c.366del	p.Ala123LeufsX12	1	1	1
	4	c.405del	p.Phe136LeufsX32	1	5	2
	4	c.418del	p.Cys140AlafsX28	1	4	2
	4	c.443G>A	p.Gly148Asp	3	5	2
	4	c.445_448dup	p.Cys150TyrfsX42	1	4	2
	4	[c.446T>A(+)c.448_450del]	p.Ile149Asn; p.Cys150del	6	8	3
	1,2,3,4	1-?_480+?del	DEL1–4	4	5	3

[a] 1 = Frameshift; 2 = non-sense; 3 = missense; 4 = large deletion; 5 = splice; 6 = in frame.

[b] Mutations not previously reported in the tricarboxylic acid (TCA) Cycle Gene Mutation Database available on http://chromium.liacs.nl/lovd_sdh/ (09/03/03). The notation of mutations was based on the guidelines described by the Human Genome Variation Society (http://www.hgvs.org/mutnomen/). The NCBI accession numbers are NM_003000.2, NM_003001.3, and NM_003002.1 for *SDHB*, *SDHC*, and *SDHD*, respectively.

our series, the initial round would have detected more than 50% (52 to 100%) of the mutations, and the first and second rounds together more than 90% (92 to 100%). This targeted strategy, rather than using extensive *SDH* genetic testing, would diminish both the cost and the delay. These recommendations require that the laboratories routinely use methods for searching for large genomic deletions that represent approximately 10% of the mutations. In our experience, MLPA and QMPSF methods are equally good for detecting large deletions; MLPA could be used first to screen the three *SDHx* genes in a single test, and QMPSF could then be performed to validate the findings. However, in our cohort of patients with a single catecholamine-secreting adrenal paraganglioma or pheochromocytoma (6), we detected only three large *VHL* deletions (2%) on 147 patients and none on the *SDHx* genes (our personal data). Those three patients had a classical syndromic presentation of von Hippel Lindau disease. To our view, in case of isolated pheochromocytoma, testing for detection of large genomic deletions is indicated only in patients with a positive family history or a syndromic presentation. Moreover, there is probably no indication to routinely test the *VHL* gene in patients with a head and neck paraganglioma without a positive family history or a syndromic presentation of von Hippel Lindau disease.

Early genetic testing is essential for cases of paraganglioma because the identification of a *SDHD* mutation is suggestive of multiple disease and, more importantly, a *SDHB* mutation indicates probable malignancy. Our large series confirms previously observed genotype-phenotype correlations for *SDHD* and *SDHB* mutations (4–6, 11, 20). *SDHD* subjects tend to have a benign head and neck disease characterized by the occurrence of multiple paragangliomas in a familial context. Importantly, the analysis of the 78 *SDHD* pedigrees did not reveal any maternal transmission of the disease or exception from the classical maternal imprinting autosomal dominant model of inheritance;

TABLE 4. Genetic data according to the presentation

		SDHs		*SDHB*			*SDHC*			*SDHD*			*SDHs*	
	Total cases	Mutation carriers	Non-mutation carriers	Point mutations[a]	Large deletions	Total mutations	Point mutations[a]	Large deletions	Total mutations	Point mutations[a]	Large deletions	Total mutations	Total point mutations[a]	Total large deletions
HN and/or TAP PGL total patient no. (%)	445	242 (54.5)	203 (45.6)	88 (36.4)	8 (3.3)	96 (39.7)	14 (5.8)	2 (0.8)	16 (6.6)	118 (48.8)	12 (5)	130 (53.7)	220 (90.9)	22 (9.1)
Single HN PGL (n = 224)														
Positive family history (%)	39	38 (97.4)	1 (2.6)	9 (23.7)	0	9 (23.7)	2 (5.3)	1 (2.6)	3 (7.9)	23 (60.5)	3 (7.9)	26 (68.4)	34 (89.5)	4 (10.5)
Age ≤35 yr (%)	49	36 (73.5)	13 (26.5)	10 (27.8)	1 (2.8)	11 (30.6)	3 (8.3)	1 (2.6)	4 (11.1)	17 (47.2)	4 (11.1)	21 (58.3)	30 (83.3)	6 (16.7)
Metastases (%)	11	7 (63.6)	4 (36.4)	6 (85.7)	0	6 (85.7)	0	0	0	1 (14.3)	0	1 (14.3)	7 (100)	0
Age >35 yr, apparently benign and sporadic at presentation (%)	150	25 (16.7)	125 (83.3)	13 (52)	0	13 (52)	5 (20)	0	5 (20)	6 (24)	1 (4)	7 (28)	24 (96)	1 (4)
Single TAP PGL (n = 99)														
Positive family history (%)	12	12 (100)	0	9 (75)	1 (8.3)	10 (83.3)	0	0	0	1 (8.3)	1 (8.3)	2 (16.6)	10 (83.3)	2 (16.7)
Age ≤35 yr (%)	42	25 (59.5)	17 (40.5)	19 (76)	3 (12)	22 (88)	0	1 (4)	1 (4)	1 (4)	1 (4)	2 (8)	20 (80)	5 (20)
Metastases (%)	24	20 (83.3)	4 (16.7)	15 (75)	5 (25)	20 (100)	0	0	0	0	0	0	15 (75)	5 (25)
Age >35 yr, apparently benign and sporadic at presentation (%)	40	6 (15)	34 (85)	6 (100)	0	6 (100)	0	0	0	0	0	0	6 (100)	0
Multiple PGLs (n = 122)														
Positive family history (%)	52	52 (100)	0	2 (3.8)	0	2 (3.8)	1 (1.9)	0	1 (1.9)	46 (88.5)	3 (5.8)	49 (94.3)	49 (94.2)	3 (5.8)
Age ≤35 yr (%)	75	71 (94.7)	4 (5.3)	15 (21.1)	0	15 (21.1)	1 (1.4)	0	1 (1.4)	53 (74.6)	2 (2.8)	55 (77.4)	69 (97.2)	2 (2.8)
Metastases (%)	14	13 (92.9)	1 (7.1)	10 (76.9)	0	10 (76.9)	0	0	0	3 (23.1)	0	3 (23.1)	13 (100)	0
Age >35 yr, apparently benign and sporadic at presentation (%)	24	19 (79.2)	5 (20.8)	3 (15.8)	0	3 (15.8)	3 (15.8)	0	3 (15.8)	11 (57.9)	2 (10.5)	13 (68.4)	17 (89.5)	2 (10.5)

HN, Head and neck; TAP, thoracic, abdominal, or pelvic; PGL, paraganglioma.

[a] Point mutations = splice-site mutation, intraexonic mutation, or small deletion or small insertion.

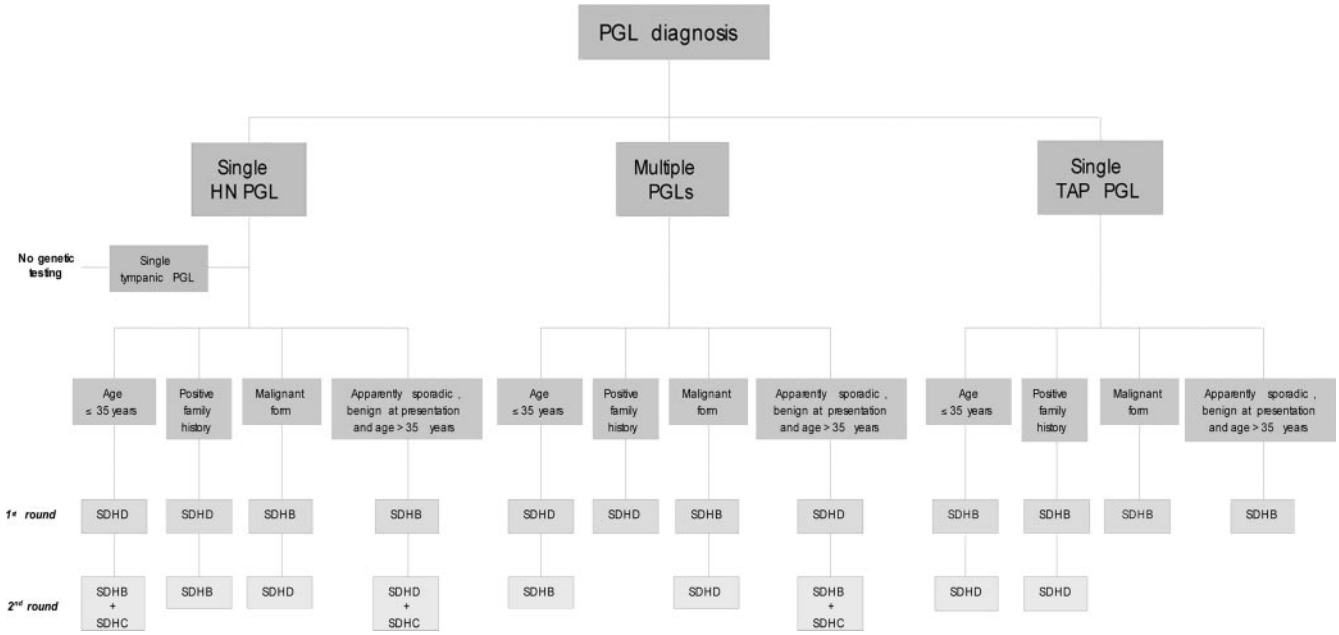

FIG. 2. Recommendations for a sequential SDH genetic testing according to the presentation. SDHD, SDHB, and SDHC genetic testing is indicated in all cases of paragangliomas except for cases of a solely tympanic location. The genetic testing should involve direct sequencing first and, if negative, subsequent investigations for large genomic deletions. TAP, Thoracic, abdominal, or pelvic; HN, head and neck; PGL, paraganglioma.

consequently, the single case report describing maternal transmission of the disease associated with *SDHD* should be interpreted with caution and even doubt (21). We recommend presymptomatic genetic testing of the child only when the father carries the *SDHD* mutation. Many *SDHB* subjects have a thoracic-abdominal or pelvic disease, in some cases malignant, without any familial history. We and others have previously demonstrated that germline mutations in the *SDHB* gene are strongly associated with malignancy and poor prognosis (6, 11, 15, 20–22). So, the identification of *SDHD* or *SDHB* mutation should lead to complete investigations before surgery to detect all paragangliomas and/or metastases (23, 24). After surgery, a tumor-free subject should be followed on an annual basis.

Fewer mutations were found in *SDHC* than in *SDHD* or *SDHB*; nevertheless, we identified as many such cases as previously described worldwide (25). We confirm that *SDHC* mutation carriers preferentially have head and neck paraganglioma, and we report on two patients with a thoracic paraganglioma. The spectrum of tumor locations in *SDHC*-related patients therefore seems to be similar to that in *SDHD*- and *SDHB*-related subjects, with the occurrence of head and neck paraganglioma, thoracic-abdominal or pelvic paraganglioma, and pheochromocytoma (25, 26). The relative numbers of large deletions (2 of 16) and point (14 of 16) mutations affecting *SDHC* are similar to those concerning the *SDHD* and *SDHB* genes. Therefore, the relative infrequency of *SDHC* mutations does not seem to be explained by a high level of large *SDHC* deletions, as was suggested by the observation of a high density of Alu elements in the *SDHC* sequence (27).

This study will help geneticists to inform asymptomatic subjects related to *SDH* mutation carriers. In front of a family with a new mutation, the genetic counseling is sustained by the quality of the genetic data given by laboratories able to routinely use different methods dedicated to prove the functionality of unknown variants. Between 2003 and 2008, 318 first-degree relatives requested familial presymptomatic genetic testing (our personal data). An investigation protocol currently ongoing has been proposed to all the *SDH*-positive carriers to evaluate the best screening methods for detecting tumors (Clinical Trial.Gov NCT00188019) and to determine better the natural history of the disease in a large cohort. This study should provide accurate penetrance data because all included subjects will be studied with the same methods. The data we report here diverge only slightly from the estimated median age given by the International SDH Consortium (4). At age 40, 62% (*vs.* 68% for the SDH International Consortium) of all *SDHD* mutation carriers and 21% (*vs.* 15%) of the *SDHB* subjects have an head and neck location. A thoracic-abdominal or pelvic paraganglioma was diagnosed in 54.2% of our *SDHB* subjects (*vs.* 69% for the SDH International Consortium), whereas only 11.5% of the *SDHD* subjects have a thoracic-abdominal or pelvic paraganglioma (*vs.* 35%) at age 60 yr. These slight discrepancies might be explained by the larger number of patients in our series: 130 *SDHD* (*vs.* 30 for the SDH International Consortium) and 96 *SDHB* (*vs.* 82) mutation carriers and because asymptomatic family members investigated after the predictive genetic testing were also considered by the SDH International Consortium. Importantly, with 8.7% (21 of 242) of the patients with paragangliomas diagnosed before the age of 18 yr, we establish that hereditary paraganglioma is a childhood onset cancer predisposition. So, predictive genetic testing could be proposed to asymptomatic children from the age of 6 yr, after an appropriate genetic counseling by genetics services in accordance with the guidelines concerning the ethical aspects of genetic testing in asymptomatic minors (28).

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