

GERM-LINE MUTATIONS IN NONSYNDROMIC PHEOCHROMOCYTOMA

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ABSTRACT

Background The group of susceptibility genes for pheochromocytoma that included the proto-oncogene *RET* (associated with multiple endocrine neoplasia type 2 [MEN-2]) and the tumor-suppressor gene *VHL* (associated with von Hippel-Lindau disease) now also encompasses the newly identified genes for succinate dehydrogenase subunit D (*SDHD*) and succinate dehydrogenase subunit B (*SDHB*), which predispose carriers to pheochromocytomas and glomus tumors. We used molecular tools to classify a large cohort of patients with pheochromocytoma with respect to the presence or absence of mutations of one of these four genes and to investigate the relevance of genetic analyses to clinical practice.

Methods Peripheral blood from unrelated, consenting registry patients with pheochromocytoma was tested for mutations of *RET*, *VHL*, *SDHD*, and *SDHB*. Clinical data at first presentation and follow-up were evaluated.

Results Among 271 patients who presented with nonsyndromic pheochromocytoma and without a family history of the disease, 66 (24 percent) were found to have mutations (mean age, 25 years; 32 men and 34 women). Of these 66, 30 had mutations of *VHL*, 13 of *RET*, 11 of *SDHD*, and 12 of *SDHB*. Younger age, multifocal tumors, and extraadrenal tumors were significantly associated with the presence of a mutation. However, among the 66 patients who were positive for mutations, only 21 had multifocal pheochromocytoma. Twenty-three (35 percent) presented after the age of 30 years, and 17 (8 percent) after the age of 40. Sixty-one (92 percent) of the patients with mutations were identified solely by molecular testing of *VHL*, *RET*, *SDHD*, and *SDHB*; these patients had no associated signs and symptoms at presentation.

Conclusions Almost one fourth of patients with apparently sporadic pheochromocytoma may be carriers of mutations; routine analysis for mutations of *RET*, *VHL*, *SDHD*, and *SDHB* is indicated to identify pheochromocytoma-associated syndromes that would otherwise be missed. (N Engl J Med 2002;346:1459-66.)

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IT is becoming increasingly apparent that tumors of a single histologic type are heterogeneous in their natural history, prognosis, and response to treatment. Tumors such as pheochromocytomas and paragangliomas may also display important molecular differences and lend themselves to genetic analysis.

It is a widespread assumption that most pheochromocytomas are sporadic and only about 10 percent are hereditary.¹ When hereditary, pheochromocytoma can be a component of multiple endocrine neoplasia type 2 (MEN-2), caused by mutations of the *RET* gene; von Hippel-Lindau disease, caused by mutations of the *VHL* gene; and, rarely, neurofibromatosis type 1.²⁻⁵ Recently, mutations of the gene for succinate dehydrogenase subunit D (*SDHD*) were identified for another related neuroendocrine disease, familial paragangliomas of the neck, or glomus tumors.⁶ *SDHD* and *SDHB* encode mitochondrial enzymes involved in oxidative phosphorylation.⁷ In a study by Heutink

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et al., all 38 affected members of five original families tested had neck paragangliomas, but none had pheochromocytomas.^{8,9} However, in our pilot study of pheochromocytomas from a small series comprising 17 unrelated patients with nonfamilial disease who showed no molecular or clinical evidence of MEN-2, von Hippel–Lindau disease, or neurofibromatosis type 1, we identified three unsuspected germ-line mutations of *SDHD*.¹⁰ In contrast, in a referral-based cohort of 19 patients with pheochromocytoma from Brazil, no mutations of *SDHD* were found.¹¹ In 2001, mutations of *SDHB* were found in three of eight families with pheochromocytoma, paraganglioma, or both.¹²

Molecular medicine makes it possible to differentiate sporadic from hereditary disease, which will affect medical management not only for the patient but also for the family. This is particularly true for inherited tumor syndromes.¹³ In the present study, we analyzed the known susceptibility genes for pheochromocytoma — *VHL*, *RET*, *SDHD*, and *SDHB* — in a large, unselected series of registry patients who presented with this tumor in order to classify them as having either truly sporadic or hereditary disease. Those who had mutations could then be reclassified as having von Hippel–Lindau disease, MEN-2, or one of the syndromes associated with pheochromocytoma and paraganglioma. In addition, we evaluated mutation status in relation to a range of clinical features to determine which, if any, can predict hereditary disease.

METHODS

Patients

Patients with pheochromocytomas have been consecutively registered in the population registries of Freiburg, Germany, and Warsaw, Poland, in accordance with the ethical standards of the respective countries. Two hundred ninety-eight consecutive, unrelated patients with histologically confirmed pheochromocytoma from whom blood-leukocyte DNA was available were enrolled. All patients provided written or oral informed consent. For the purposes of registration, all cases of pheochromocytoma were included except those discovered by clinical or genetic screening of persons without symptoms of illness, in order to minimize ascertainment bias toward hereditary cases. For the purposes of this study, we excluded 11 patients with neurofibromatosis type 1, since all these patients had classic cutaneous lesions and could be easily given a diagnosis of a syndromic condition without molecular genetic analyses. We further excluded 14 patients with a family history (9 with tumors related to von Hippel–Lindau disease, and 5 with tumors related to MEN-2). DNA samples were available from 70 percent of the patients in the Freiburg registry and 88 percent of the patients in the Warsaw registry. Thus, 271 eligible registrants entered our study.

Of the 271 patients, 241 presented with pheochromocytomas only, whereas 8 presented with both pheochromocytomas and functioning paragangliomas. Twenty-two presented with paragangliomas only. Paragangliomas that originate in the sympathetic nervous system are most commonly found in the retroperitoneum but can also occur in the thorax as catecholamine-secreting, “functioning” extra-adrenal pheochromocytomas (and are frequently included in the term “pheochromocytomas,” which we use here). Paragangliomas that originate in the parasympathetic nervous system can occur adjacent to the aortic arch, neck, and skull base as local “nonfunction-

ing” masses, also called glomus tumors or chemodectomas. Unless the location of the tumor is germane, we will refer to our patients as presenting with pheochromocytoma.

Molecular Genetic Analyses

All eight exons of *SDHB*, all four exons of *SDHD*, all three exons of *VHL*, and exons 10, 11, and 13 through 16 of *RET* were examined by analysis of single-strand conformation polymorphisms and direct sequencing, as previously described.^{10,12,14–18}

When a patient with a germ-line mutation was identified, his or her consenting parents were investigated for the presence of the mutation; this analysis enabled us to trace the disease and the mutation back to the previous generation. If neither parent carried the index patient’s mutation, we confirmed paternity using standard microsatellite fingerprinting. Genomic DNA samples from 300 anonymous, healthy blood donors matched with the registry patients for race (white) and region were analyzed as controls.

Clinical Studies

One of us performed or reviewed the clinical evaluation (personal and family history and physical examination) and medical records (detailed personal and family history, physical examination, and biochemical imaging studies). Family history was also updated at the time of blood sampling; final updates of all clinical data were performed through December 1, 2001.

Patients who were clinically or genetically identified in the present study as having a hereditary pheochromocytoma syndrome underwent clinical evaluation and surveillance for MEN-2, von Hippel–Lindau disease, and syndromes associated with pheochromocytoma and paraganglioma. The clinical screening program included measurement of serum calcitonin levels after stimulation with pentagastrin and measurement of serum parathyroid hormone levels for MEN-2; magnetic resonance imaging (MRI) of the central nervous system, MRI or computed tomography (CT) of the abdomen, and retinoscopy for von Hippel–Lindau disease²; and MRI of the abdomen, thorax, and neck for syndromes associated with pheochromocytoma and paraganglioma. The clinical diagnosis of MEN-2 required the occurrence of pheochromocytoma and medullary thyroid carcinoma.⁴ In addition to pheochromocytoma at presentation, the diagnosis of von Hippel–Lindau disease required at least angioma of the retina or hemangioblastoma of the central nervous system in the index patient or a first-degree relative.¹⁹ The diagnosis of neurofibromatosis type 1 was made according to standard criteria.⁵

Pheochromocytomas were classified according to number (solitary or multiple), location (adrenal or extraadrenal), and pathological findings (benign or malignant). Distant metastases or infiltration of surrounding tissue was required to designate a pheochromocytoma as malignant.¹

Statistical Analysis

For the comparison of rates from small samples, Fisher’s (two-tailed) unpaired exact test was used; for larger groups, the standard two-sided chi-square test was used. P values less than 0.05 were considered to indicate statistical significance.

RESULTS

A total of 271 patients (155 female and 116 male; age range, 4 to 81 years; mean age, 40 years) with nonsyndromic pheochromocytoma and without a family history of the disease were enrolled in the study. We identified 66 patients with deleterious germ-line mutations (24 percent); 13 had mutations of *RET*, 30 mutations of *VHL*, 11 mutations of *SDHD*, and 12 mutations of *SDHB* (Table 1).

TABLE 1. AGE OF THE PATIENTS AND TYPE OF TUMOR AT PRESENTATION ACCORDING TO GENETIC STATUS.

VARIABLE	MEN-2 (N=13)	VON HIPPEL- LINDAU DISEASE (N=30)	SDHD MUTATION-ASSOCIATED PHEOCHROMOCYTOMA- PARAGANGLIOMA SYNDROME (N=11)	SDHB MUTATION-ASSOCIATED PHEOCHROMOCYTOMA- PARAGANGLIOMA SYNDROME (N=12)	HEREDITARY DISEASE (N=66)	NONSYNDROMIC DISEASE (N=205)	TOTAL (N=271)	P VALUE*
Age at presentation (yr)								
Mean	36.4	18.3	28.7	25.6	24.9	43.9	39.3	<0.001
Range	21-50	5-49	5-59	12-48	5-59	4-81	4-81	
Age at onset ≤18 yr (no.)	0	20	3	4	27	21	48	<0.001
Type of tumor (no.)								
Multifocal	5	12	4	0	21	5	26	<0.001
Extraadrenal	0	4	4	6	14	16	30	0.006

*The P values are for the comparison of hereditary disease with nonsyndromic diseases.

Frequency Distribution and Types of Mutations

Thirteen unrelated patients (5 percent) were found to have seven germ-line mutations of *RET* (Table 2). These were missense mutations, like the majority of mutations of *RET* associated with MEN-2 to date.²¹ Haplotype analysis to exclude a founder effect was inconclusive, but relatedness seemed unlikely on re-evaluation of the family histories.

The mutations of *VHL* in 30 patients (11 percent) comprised 3 nonsense and 19 missense mutations (Table 2). Among these 22 distinct mutations, 4 were novel. We had access to the DNA of both parents of four patients with diagnoses of pheochromocytoma (whose ages were 5, 7, 8, and 16 years). No genetic or clinical evidence of von Hippel-Lindau disease could be found in the parents, and microsatellite analysis at five different informative loci in the four families confirmed paternity. Thus, these four cases represent spontaneous germ-line mutations of *VHL*. Therefore, relationship of each carrier pair with the G490A and the G695A complementary DNA (cDNA) mutations (Table 2) has been excluded. The C712T cDNA mutation represents a well-known "hot spot,"²² and extensive pedigree evaluation makes it unlikely that the patients with the A680T cDNA mutation are related. It should be noted, however, that shared haplotypes usually denote ancient population-based founder effects instead of closer intermarriages that can be identified through the family history.

Eleven patients (4 percent of the 271) had seven different mutations of *SDHD*, three of which were novel (Table 2). Six mutations cause truncation of the putative protein, whereas one leads to a substitution of one amino acid. There were two different recurrent mutations. Haplotype analysis was inconclusive because of the small numbers, but pedigree information made relationship unlikely.

Twelve patients (4 percent) were found to have nine

distinct, novel mutations of *SDHB* (Table 2). There were three recurrent mutations, none of which occurred in shared haplotypes; extensive pedigree evaluation also demonstrated that it was unlikely that any of the three carrier pairs were related.

None of the seven mutations of *SDHD* or the nine mutations of *SDHB* found in our patients were found in 600 control chromosomes from the 300 blood donors.

Clinical Presentation and Follow-up of Carriers

The age at the onset of symptoms was statistically lower in all carriers of mutations than in patients with sporadic disease, who were operationally defined as negative for mutations of any of the four susceptibility genes. Seventy percent of the patients who presented at the age of 10 or younger had germ-line mutations, and this percentage decreased steadily with increasing age to 0 percent among patients who presented after the age of 60 (Table 3). However, 139 registrants presented with pheochromocytoma after the age of 40, 11 of whom had mutations (8 percent). Only five of these patients had clinical findings that retrospectively suggested a hereditary pheochromocytoma. Mutations of *VHL* were present in 42 percent of all those who presented at age 18 or younger (20 of 48) and 74 percent of all patients with mutations who presented at age 18 or younger (20 of 27); 77 percent of those found to have mutations of *VHL* presented before the age of 20 (Table 3). In contrast, 9 of the 23 patients found to have germ-line mutations of *SDHD* and *SDHB* (39 percent) presented after the age of 30.

At initial presentation, 45 of 66 probands with mutations (68 percent) had only one tumor (Table 1). Multiple pheochromocytomas, however, were statistically more frequent among patients with mutations than among patients without mutations (21 of 66

TABLE 2. GERM-LINE MUTATIONS IN THE FOUR GENES DETECTED IN THE SERIES OF PATIENTS WITH PHEOCHROMOCYTOMA.

GENE	MUTATION (cDNA NUCLEOTIDE)	CONSEQUENCE (AMINO ACID)	EXON	INDEPENDENT
				CASES
				no.
<i>RET</i>	T1900C	Codon 634, Cys to Arg	11	4
	T1900G	Codon 634, Cys to Gly	11	1
	G1901A	Codon 634, Cys to Tyr	11	3
	G1901C	Codon 634, Cys to Ser	11	1
	G1901T	Codon 634, Cys to Phe	11	1
	C1902G	Codon 634, Cys to Trp	11	2
	A2372T	Codon 791, Tyr to Phe	13	1
	<i>VHL*</i>	T406G	Codon 65, Ser to Ala	1
C416G		Codon 68, Ser to Trp	1	1
G452A		Codon 80, Ser to Asn	1	1
G490A†		Codon 93, Gly to Ser	1	2
G490T		Codon 93, Gly to Cys	1	1
G491T‡		Codon 93, Gly to Cys	1	1
G493T		Codon 94, Glu to Stop	1	1
T505C		Codon 98, Tyr to His	1	1
C532G†‡		Codon 107, Arg to Gly	1	1
C570G		Codon 119, Phe to Leu	2	1
GC577, 578AT‡		Codon 122, Ala to Ile	2	1
T620G		Codon 136, Phe to Cys	2	1
T679A‡		Codon 156, Tyr to Asn	3	1
A680G		Codon 156, Tyr to Cys	3	2
G695A†		Codon 161, Arg to Gln	3	2
G695C		Codon 161, Arg to Pro	3	1
C703T		Codon 164, Gln to Stop	3	1
C712T†		Codon 167, Arg to Trp	3	6
G713A		Codon 167, Arg to Gln	3	1
C775G		Codon 188, Leu to Val	3	1
C796T	Codon 195, Gln to Stop	3	1	
T806A	Codon 198, Leu to Gln	3	1	
<i>SDHD</i>	G14A	Codon 5, Trp to Stop	1	1
	C33A	Codon 11, Cys to Stop	1	4
	36,37 del TG‡	Frame shift	1	1
	52+2 (IVS 1+2) T/G§	Splice defect	1	1
	C112T	Codon 38, Arg to Stop	2	2
	G274T‡	Codon 92, Asp to Tyr	3	1
	C361T‡	Codon 121, Gln to Stop	4	1
	<i>SDHB</i>	C213T‡	Codon 27, Arg to Stop	2
221insCAG‡		Codon 29, insertion of Gln	2	1
C270G‡		Codon 46, Arg to Gly	2	2
G436A‡		Codon 101, Cys to Tyr	4	2
T708C‡		Codon 192, Cys to Arg	6	1
G721A‡		Codon 196, Cys to Tyr	6	1
847delTCTC‡		Frame shift	7	2
C859A‡		Codon 242, Arg to His	7	1
C881A‡	Codon 249, Cys to Stop	7	1	

*Some of these mutations have been described previously.²⁰

†The mutation is spontaneous.

‡The mutation is novel.

§IVS denotes intervening sequence.

[32 percent] vs. 5 of 205 [2 percent], $P < 0.001$). However, multifocal tumors, as compared with solitary tumors, may be gene-specific: no patients with mutations of *SDHB* presented with multifocal disease, whereas 40 percent of those with mutations of *VHL* had multifocal disease. Twenty-eight percent of the patients with mutations of *VHL*, *SDHD*, and *SDHB*

had extraadrenal tumors, as compared with 8 percent of patients without mutations ($P = 0.006$).

We also identified classic syndrome-associated lesions at presentation and at final follow-up. Of 13 patients who were positive for mutations of *RET*, none had clinical evidence of medullary thyroid carcinoma at presentation, but medullary thyroid carcinoma de-

TABLE 3. AGE AT PRESENTATION OF PATIENTS WITH MUTATIONS OR SPORADIC DISEASE.

GENETIC STATUS	AGE AT PRESENTATION						
	0–10 YR (N=10)	11–20 YR (N=47)	21–30 YR (N=31)	31–40 YR (N=44)	41–50 YR (N=56)	51–60 YR (N=51)	61–81 YR (N=32)
Mutated gene (no.)							
<i>VHL</i>	6	17	2	3	2	0	0
<i>RET</i>	0	0	4	4	5	0	0
<i>SDHD</i>	1	2	3	3	1	1	0
<i>SDHB</i>	0	5	3	2	2	0	0
No mutation (no.)	3	23	19	32	46	50	32
Hereditary disease (%)*	70	51	39	27	18	2	0

*Values are the percentages of hereditary cases found by molecular genetic methods.

veloped in 12 during the follow-up period. Among 30 carriers of mutations of *VHL*, 5 also subsequently had other features associated with von Hippel–Lindau disease, such as hemangioblastoma of the central nervous system or eye, pancreatic cysts, islet-cell tumors, or renal-cell carcinomas, during follow-up. In total, 10 patients were found to have associated lesions during follow-up. Of 23 carriers of *SDHD* or *SDHB* mutations, none had glomus tumors at presentation, but these tumors developed in 4 patients during follow-up (Fig. 1). The majority of medullary thyroid carcinomas and glomus tumors were detected by screening.

Because of our exclusion criteria, none of the 66 patients who were positive for mutations presented with a family history of syndrome-specific tumors. However, among patients with mutations of *RET*, six had a positive family history at the final follow-up. Similarly, 12 carriers of mutations of *VHL* had a positive family history at follow-up. None of the 23 patients found to have germ-line mutations of *SDHD* or *SDHB* had a positive family history at initial presentation. Even at follow-up, only four had family members who had been found to have clinical disease. There was a delay of up to 35 years before relatives began to have symptoms, and for 10 of 23 patients, the family history became positive only when active clinical screening was performed. Forty-two of the 66 probands with mutations (64 percent) had only one pheochromocytoma and no associated syndrome-specific lesion. Over 80 percent of patients with mutations of *SDHD* (7 of 11) or *SDHB* (12 of 12) presented with one pheochromocytoma, no family history, and no feature of associated syndromes. In contrast, this was true in about half of the patients with mutations of *RET* (8 of 13) or *VHL* (15 of 30).

DISCUSSION

Our systematic clinical and molecular evaluation of 271 unrelated patients who presented with nonsyn-

dromic pheochromocytoma revealed that 66 (24 percent) had a hereditary predisposition to von Hippel–Lindau disease, MEN-2, or the syndromes associated with pheochromocytoma and paraganglioma on the basis of newly discovered mutations in the *VHL*, *RET*, *SDHD*, or *SDHB* gene. Among the 66 patients with mutations, 45 percent had germ-line mutations of *VHL*, 20 percent had mutations of *RET*, and 17 and 18 percent had mutations of two newly identified genes, *SDHD* and *SDHB*. Currently, 64 percent of all probands found to have hereditary disease were identified with the use of molecular testing of *VHL*, *RET*, *SDHD*, and *SDHB* and had no family history, solitary disease, and no associated signs and symptoms at presentation.

Several previous studies, limited by small size, hinted that certain subgroups of patients with pheochromocytoma might have a higher risk of hereditary disease — those with bilateral or multifocal tumors, those who are relatively young at presentation, or both.^{2,4,23,24} Our registry-based study addresses these issues by its complete or nearly complete identification of virtually all cases of bilateral tumors, cases of extraadrenal pheochromocytoma (paraganglioma), and cases with early onset. We can state confidently that 84 percent of all multifocal tumors (including bilateral tumors) and 59 percent of pheochromocytomas with onset at the age of 18 years or younger were found to be hereditary. Our results suggest that extraadrenal pheochromocytoma may be a striking feature of hereditary disease ($P=0.006$). When extraadrenal disease is found either in isolation or with adrenal pheochromocytoma, the molecular differential diagnosis, in descending order, includes mutations of *SDHB* (in 50 percent of cases), *SDHD* (36 percent), and *VHL* (17 percent) but not *RET*.

A partial explanation for the high frequency of hereditary pheochromocytoma without a family history of disease might include spontaneous mutation in

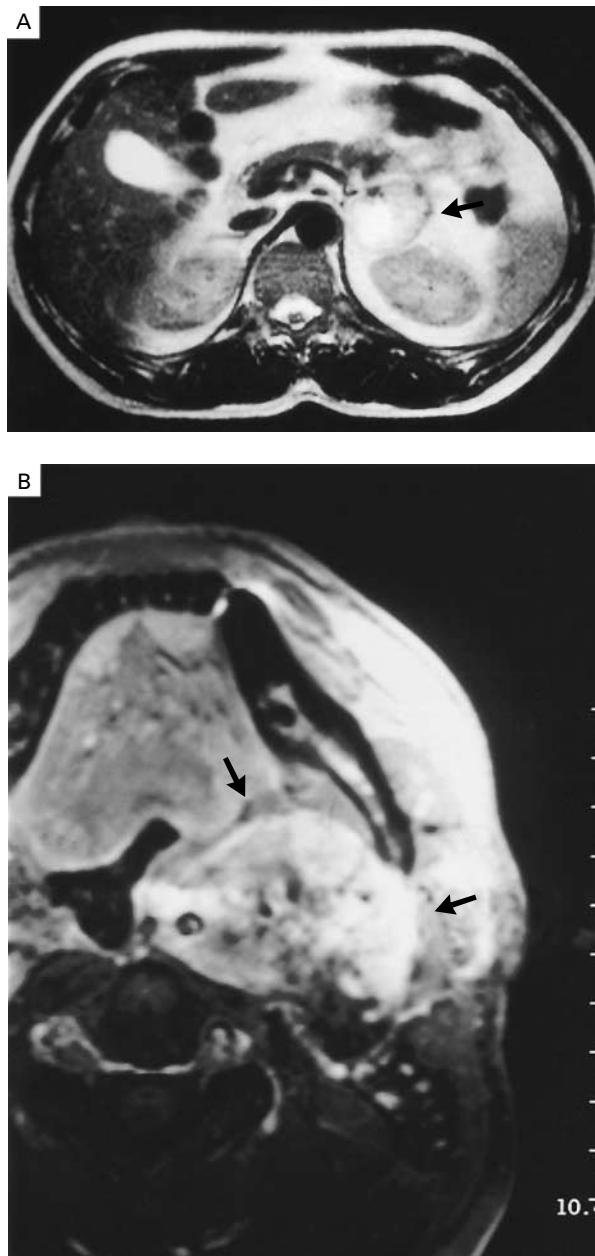


Figure 1. Pheochromocytoma of the Left Adrenal Gland and Glomus Tumor of the Left Carotid Body in a Carrier of a Mutation of *SDHD*.

The pheochromocytoma became symptomatic five years earlier than the glomus tumor. In Panel A, transverse T_2 -weighted abdominal MRI shows a hyperintense left adrenal pheochromocytoma (arrow). In Panel B, contrast-enhanced transverse cervical MRI (T_1 -weighted images with spectral fat saturation) reveals a contrast-enhancing left cervical glomus tumor (arrows).

one of the susceptibility genes, decreased penetrance, and maternal imprinting. In our registry, spontaneous mutations in *VHL* accounted for 13 percent of cases of hereditary von Hippel–Lindau disease. Penetrance is known to be relatively high (approximately 70 percent by the age of 70) among patients with MEN-2 and von Hippel–Lindau disease, overall.^{25,26} The penetrance of mutations of *SDHD* and *SDHB* is not well known because they are newly identified genes. Preliminary figures from this registry suggest a relatively high penetrance. Familial glomus tumors due to mutations of *SDHD* are known to be maternally imprinted.^{6,9} Overall, therefore, pheochromocytomas in patients without family histories are due to spontaneous mutations, decreased penetrance, or maternal imprinting, although other causes such as gene–gene interactions and gene–environment interactions may be possible.

Genetic testing can be a powerful aid to the identification of a syndrome in such cases. For example, our study suggests that a patient with pheochromocytoma who has mutations of *SDHD* or *SDHB* has an approximately 20 to 30 percent likelihood of already having glomus tumors or of having glomus tumors develop. Since such tumors are difficult to treat when advanced (Fig. 1), it is reasonable to speculate that molecular identification of a mutation in one of these two genes could lead to surveillance, early diagnosis of tumors, and more effective treatment. In our study, 68 percent of patients found to have germ-line mutations presented with solitary tumors. It is the standard of care for clinical cancer geneticists to offer genetic testing to patients with a minimal a priori risk of mutation of 10 percent.¹³ This figure can be lowered if the technique of mutation analysis is straightforward and cost effective and the alteration in medical management saves lives, as is the case for testing for mutations of *RET*.²⁷ Our registry-based study has demonstrated that the a priori risk of finding a mutation of *VHL*, *RET*, *SDHD*, or *SDHB* in unrelated patients who present with pheochromocytoma not only meets but exceeds both of these criteria, even in the case of patients who are over the age of 40 at presentation.

In our opinion, although it has not yet been demonstrated by a clinical trial, the identification of a new case of hereditary pheochromocytoma on the basis of the identification of the disease-associated mutation should prompt genetic testing of all first-degree relatives of the carrier to determine the presence or absence of the family-specific mutation. Since disease is likely to develop in virtually all patients with a family-specific mutation, it seems reasonable to subject such patients to lifelong surveillance, prophylactic surgery, or both, depending on the precise genetic diagnosis.

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APPENDIX

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