

*Advances in Brief*

## Frequent Germ-Line Succinate Dehydrogenase Subunit D Gene Mutations in Patients with Apparently Sporadic Parasympathetic Paraganglioma

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**Abstract**

**Purpose:** Recently, familial paraganglioma (PGL) was shown to be caused by mutations in the gene encoding succinate dehydrogenase subunit D (*SDHD*). However, the prevalence of *SDHD* mutations in apparently sporadic PGL is unknown. We studied the frequency and spectrum of germ-line and somatic *SDHD* mutations in patients with parasympathetic PGL.

**Experimental Design:** We studied 57 unselected patients who developed parasympathetic PGLs ( $n = 105$  tumors) and who were treated between 1987 and 1999 at the Erasmus MC (Rotterdam, the Netherlands). Thirty-eight (67%) of these patients ( $n = 51$  tumors) lacked a family history of parasympathetic PGL. We used conformation-dependent gel electrophoresis and sequence determination analysis of germ-line and tumor DNA to identify *SDHD* mutations. We compared the clinical and molecular characteristics of sporadic and hereditary PGLs.

**Results:** Three different *SDHD* germ-line mutations were identified in 32 of the 57 (56%) patients. These included 19 of 19 (100%) patients with familial PGL and also 13 of 38 (34%) patients with apparently sporadic PGL. All three mutations were characterized as missense mutations (D92Y, L95P, and L139P) in highly conserved regions of the *SDHD* gene and were not observed in 200 control alleles. No somatic mutations were found.

**Conclusions:** Germ-line mutations of the *SDHD* gene are present in a significant number of patients with appar-

ently sporadic parasympathetic PGL. Somatic *SDHD* mutations do not play a significant role in the sporadic form of this tumor. Genetic testing for *SDHD* germ-line mutations should be considered for every patient presenting with this tumor, even if a personal or family history of PGL is absent, to allow appropriate clinical management.

**Introduction**

Parasympathetic PGLs<sup>3</sup> are highly vascularized, slow-growing tumors of parasympathetic ganglia, mostly of the head and neck region. The annual incidence is estimated at 1 in 30,000, and the tumors typically present in the fourth or fifth decade (1, 2). The tumor most commonly presents as a painless neck mass or with symptoms due to compression and damage of the surrounding structures. Depending on the anatomical localization, cranial nerve damage may cause bradycardia, hoarseness, or hearing loss. Approximately 1% of the tumors produce catecholamines. Progression to malignancy occurs in 2–10% of cases and can only be determined by detection of metastatic spread, particularly to the lymph nodes and lungs. There are currently three treatment strategies: (a) surgery; (b) radiation therapy; or (c) observation (3). Because tumor growth as well as surgery can cause disabling loss of function, preoperative estimation of tumor growth is an important parameter influencing clinical management. A “wait and scan” approach is thus often considered, although surgical resection remains the mainstay of the curative treatment of parasympathetic PGLs (4, 5).

Familial occurrence is observed in a significant minority (approximately 20%) of cases, with an autosomal dominant mode of transmission with incomplete and age-dependent penetrance (6–8). The female predominance in sporadic PGL with equal sex distribution in familial patients and an almost exclusively paternal inheritance pattern in these families suggest maternal imprinting of the disease gene (9). Clinical features that suggest a genetic predisposition in a given patient include bilateral or multiple PGLs and an unusually young age (before the third decade) at presentation. Linkage analysis and loss of heterozygosity studies in unrelated and multigenerational families with hereditary PGL (Mendelian Inheritance Man number 168,000) have provided evidence for the existence of two distinct PGL susceptibility genes on chromosome 11, *PGL1* at 11q23 and *PGL2* at 11q13, both of which are thought to be maternally imprinted (10, 11). Recently, the *SDHD* gene, which maps to the *PGL1* locus, has been implicated as the putative disease gene because inactivating germ-line *SDHD* mutations

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<sup>3</sup> The abbreviations used are: PGL, paraganglioma; SDHD, succinate dehydrogenase subunit D; SRS, somatostatin receptor scintigraphy; MC, Medical Center; SSCP, single-strand conformational polymorphism; SNP, single-nucleotide polymorphism.

have been detected in PGL families (12). *SDHD* germ-line mutations were also recently described in a few cases of familial pheochromocytoma, and to date, only one sporadic mutation has been reported (13).

The *SDHD* gene, which comprises four exons and three introns extending over 19 kb, encodes a 159-amino acid protein. This protein constitutes the small subunit (cybS) of cytochrome *b* in the mitochondrial enzyme complex II (succinate-ubiquinone oxidoreductase) and plays an important role in both the citric acid cycle and the aerobic respiratory chain (14). Recently, it has been demonstrated that germ-line mutations in succinate dehydrogenase subunit C (*SDHC*) and succinate dehydrogenase subunit B (*SDHB*), encoding two other components of complex II, also predispose individuals to hereditary PGL (15, 16).

Whether genetic alterations of the *SDHD* gene play a role in the pathogenesis of sporadic parasympathetic PGL is presently unknown. Also, the prevalence of germ-line *SDHD* mutations among apparently sporadic PGL patients has not been studied. Further knowledge of the contribution and nature of gene mutations involved in PGL tumorigenesis could provide early diagnosis and allow accurate genetic counseling in affected families. Therefore, we assessed the frequency and type of *SDHD* mutations in an unselected series of patients with parasympathetic PGLs treated at our hospital, including both familial and apparently sporadic cases.

## Patients and Methods

**Patients.** Individuals who received a diagnosis of parasympathetic PGL between May 1987 and December 1999 were identified by retrospectively reviewing the records of the Pathology Department at the Erasmus MC. Of the 89 PGL patients eligible to participate, specimens and constitutional DNA from 57 patients were available for retrieval and testing. Information on medical and family histories was obtained by direct interviews and by review of the medical charts. The presence of multiple tumors was assessed by review of the radiology reports of SRS and/or magnetic resonance imaging. From 48 patients, SRS and/or magnetic resonance imaging reports were available [41 patients (72%) underwent SRS]. Of the nine patients without such information, four had histopathological diagnoses of bilateral or multiple tumors. The average follow-up period was 52 months (range, 1–218 months), with no significant difference between familial and apparently sporadic patients.

A total of 105 parasympathetic PGLs was observed in the 57 patients, of which 78 tumors and matched normal tissues were available for analysis. After coupling of the clinical information to the pathology specimen, both patient information and DNA samples were anonymized to preserve the confidentiality of the patients and in accordance with the Erasmus MC guidelines for studies involving patient data and tissues. A tumor was considered sporadic if the patient did not know any relative with PGL.

Control DNA consisted of 100 encoded peripheral blood samples from persons residing in the same geographic area, randomly selected from the blood bank (Bloedbank ZWN Rotterdam, the Netherlands).

In addition, 13 PGLs from 13 patients residing in other

geographic areas (from hospitals in the south of the Netherlands and Belgium) were investigated for exon 3 *SDHD* mutations.

**Clinical Database.** A collective database of clinical and molecular features was prepared. The date of the patient's first biopsy or resection with a histopathological diagnosis of parasympathetic PGL was recorded as the date of diagnosis. The age of onset was defined as the age at which the patient first experienced symptoms. Three patients (two women and one man) were related to one another, and an additional two patients belonged to another family. For each patient, we recorded the place of birth, age of onset and diagnosis, clinical history, the patient's family history with respect to parasympathetic PGL, the laterality/multifocality of the tumors, and the presence of any metastases.

**DNA Preparation and SSCP Analysis.** Fresh frozen or formalin-fixed, paraffin-embedded tumor and normal tissues from all patients, including 78 of the 105 tumors, were retrieved from the archives of the Pathology Department of the Erasmus MC. Paraffin blocks containing tumor and normal tissues were obtained from 13 patients residing in other areas [kindly provided by Prof. Dr. E. Van Marck (Department of Pathology, University Hospital Antwerp, Antwerp, Belgium), Dr. G. van Lijnschoten (Society for Pathological Anatomy and Medical Microbiology, Eindhoven, the Netherlands), and Dr. A. P. de Bruïne (Department of Pathology, University of Maastricht, Maastricht, the Netherlands)].

From each specimen, regions containing at least 80% neoplastic cells were selected. DNA from 12 freshly frozen tumors was isolated using the D-5000 Pure gene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's recommendations. DNA from 66 paraffin-embedded tumors and all normal tissues was extracted by standard detergent-proteinase K lysis, followed by phenol-chloroform extraction and ethanol precipitation, as described previously (17).

The entire open reading frame of the *SDHD* gene and all exon-intron boundaries were investigated with the PCR primers described previously (12). PCR amplification of tumor DNA and matched normal DNA was performed in 15- $\mu$ l reaction mixtures containing 1.5 mM MgCl<sub>2</sub>; 10 mM Tris-HCl; 50 mM KCl; 0.02 mM dATP; 0.2 mM dGTP, dTTP, and dCTP; 0.8  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]dATP (Amersham, Buckinghamshire, United Kingdom); 20 pmol of each sense and antisense primer; and 1 unit of Taq DNA polymerase (Amplitaq Gold; Perkin-Elmer, Norwalk, CT). The amplification profile consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55 for 60 s, and extension at 72°C for 60 s. A final extension step was carried out at 72°C for 10 min. Electrophoresis of PCR products was carried out overnight at 8 W on nondenaturing gels containing 8% polyacrylamide (49:1) and 10% (v/v) glycerol. For the exon 4 amplicons, electrophoresis was performed on an 8% polyacrylamide gel without glycerol for 6 h at 4°C and 20 W. The gels were dried and exposed to X-ray film overnight at -70°C.

**DNA Sequencing.** For each variant pattern identified by SSCP analysis, two independent genomic DNA samples were amplified for direct sequencing, or the aberrant PCR product was cloned and reamplified with the original primer pair. These products were bidirectionally sequenced using Applied Biosys-

Table 1 Clinical characteristics of 57 PGL patients evaluated for *SDHD* mutations

Characteristic	All patients (N = 57)	Patients with positive family history <sup>a</sup> (N = 19)	Patients with negative family history <sup>a</sup> (N = 38)	P <sup>b</sup>
Patient				
Sex [no. (%)]				0.56
Male	21 (37)	8 (42)	13 (34)	
Female	36 (63)	11 (58)	25 (66)	
Mean age of onset ± SE (yrs)	42.4 ± 12.4	37.3 ± 8.2	45.0 ± 14.1	0.04
Mean follow-up time [months (range)]	52 (1–218)	57 (1–166)	49 (1–218)	0.56
Tumor focality [no. (%)]				<0.0001 <sup>c</sup>
Single PGL	30 (53)	2 (10)	28 (74)	
Recurrence	7 (12)		7 (18)	
Bilateral carotid body	10 (17)	7 (37)	3 (8)	
Multiple	17 (30)	10 (53)	7 (18)	
Site of tumor [no. (%)]	N = 105	N = 51	N = 54	
Carotid body	55 (52)	33 (64)	22 (41)	0.17
Vagal nerve	17 (16)	8 (16)	9 (17)	
Tympanic nerve	15 (14)	3 (6)	12 (22)	0.035
Jugular nerve	13 (13)	6 (12)	7 (13)	
Mediastinal	4 (4)	1 (2)	3 (5)	
Spinal cord	1 (1)	0 (0)	1 (2)	

<sup>a</sup> Family history data as reported at the time of the original interview.

<sup>b</sup> We used the  $\chi^2$  test to compare all variables except mean age at onset and mean follow-up time, for which we used an unpaired *t* test.

<sup>c</sup> *P* is for comparison of single PGL with bilateral and multiple PGL.

tems Taq DyeDeoxy terminator cycle sequencing (Baseclear, Leiden, the Netherlands).

**Statistics.** Correlations between a specific *SDHD* mutation and clinical features were tested by use of the  $\chi^2$  test or an unpaired *t* test. *P*s < 0.05 were considered statistically significant.

## Results

**Patient Characteristics.** The characteristics of the patients and their tumors are shown in Table 1. The group of 19 patients with a positive family history of PGL showed a roughly equal sex distribution (42% male *versus* 58% female), whereas there was a female predominance in patients without a family history of PGL (34% male *versus* 66% female). The mean age at onset was earlier in patients with a positive family history (*P* = 0.04). The majority of patients with a single PGL tumor had no family history, and bilateral carotid body tumors and multiple tumors were generally associated with a positive family history (*P* < 0.0001).

The carotid body was the most common site of origin (52%) and comprised 33 of 51 (64%) tumors in familial PGL patients. Tympanic nerve PGLs occurred more frequently in apparently sporadic patients (*P* = 0.035), with other sites being equally distributed between the two groups.

None of the patients had evidence of metastatic disease at the end of our study. Of the 57 patients, only 1 young patient (1.8%) had evidence of a malignant PGL (tumors cells were detected in one lymph node after total resection of a carotid body tumor). After 141 months of follow-up, she is alive and well.

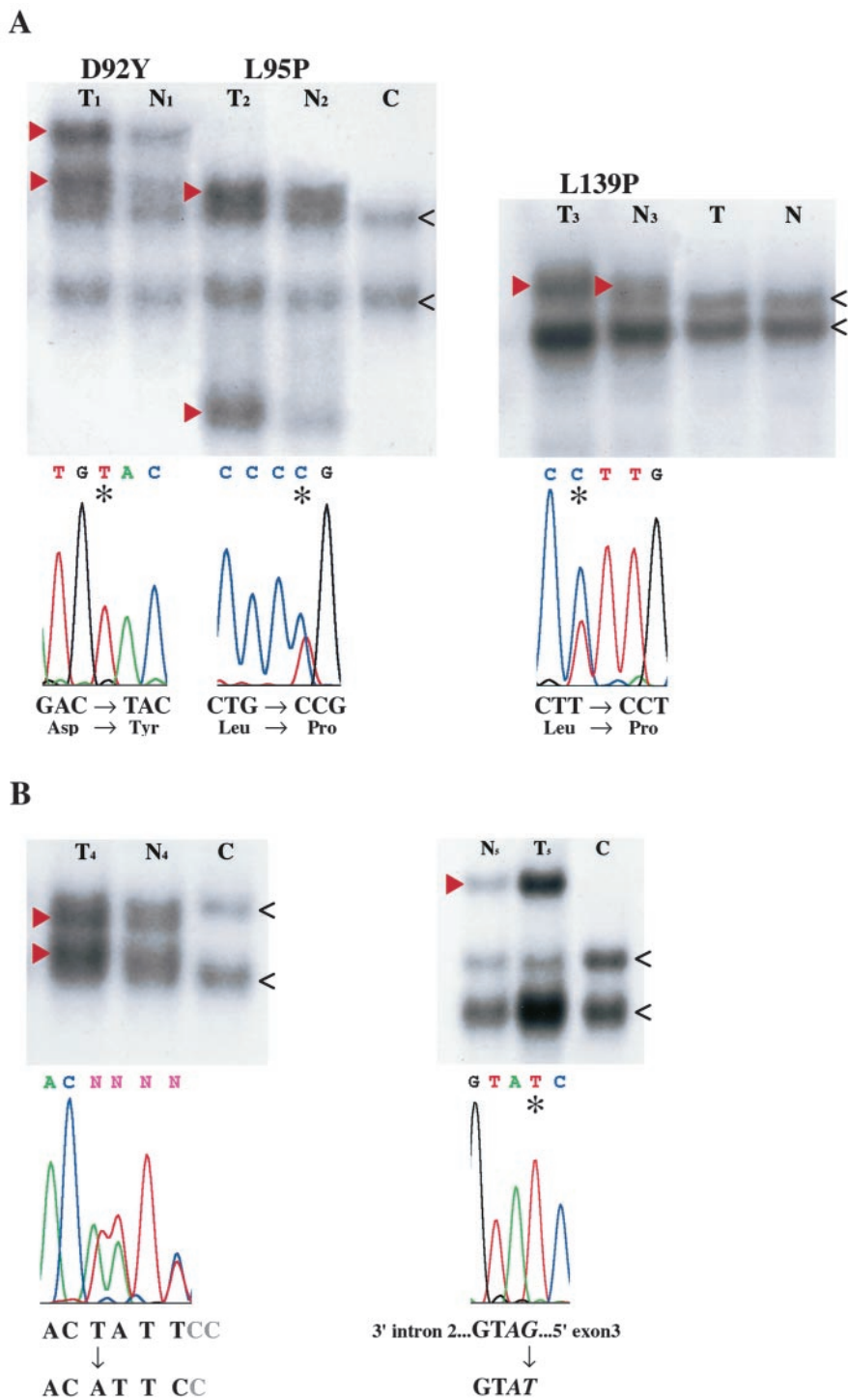
Seven patients developed a recurrence after resection of a single PGL, which was either a tympanic, jugular, or vagal tumor. None of these patients had a positive family history.

**Identification of *SDHD* Gene Mutations.** SSCP analysis revealed four different aberrant patterns, which were present in 49 of 78 (63%) PGL specimens. In each instance, the same SSCP variant was also found in the germ-line DNA of the patient (totaling 33 of 57 patients; 58%). By sequence analysis, the aberrant patterns, which were all located in exon 3 and exon 4, were found to correspond to three different missense *SDHD* mutations and one SNP. All three missense mutations were located in highly conserved regions and were absent from the 200 control alleles. The specific D92Y missense mutation was observed in 22 patients, the L95P mutation was observed in 9 patients, and the L139P mutation was observed in 1 patient. Altogether, germ-line *SDHD* missense mutations were identified in 32 of 57 (56%) patients.

The SNP, which does not result in an amino acid substitution in the *SDHD* protein, involved a change in codon 68 (AGC→AGT). This sequence alteration was also observed in one of the 200 control alleles and is therefore regarded as an uncommon polymorphism apparently not associated with hereditary PGL.

Examples of SSCP analysis and sequence determination in patients with *SDHD* missense mutations are shown in Fig. 1A.

**Patient Characteristics Associated with *SDHD* Mutations.** A germ-line *SDHD* mutation was associated with a positive family history for PGL in 19 of 32 (59%) patients but was associated with a negative family history for PGL in 13 of 32 (41%) patients (Table 2). The mean age of onset in patients harboring a germ-line missense mutation was 40.3 ± 11.4 years, compared with 44.9 ± 14.2 years in patients without a *SDHD* mutation, which is not a significant difference. The difference in age of onset also did not differ significantly among the specific mutations. Eighty-eight percent of patients without a *SDHD* mutation had a single PGL tumor, whereas in patients with



*Fig. 1. SDHD alterations identified in PGL patients by SSCP analysis and direct sequencing. SSCP patterns of the tumor (T) and germ-line (N) DNA are shown from all SDHD alterations. C, normal control sample. A shows the three missense mutations (D92Y, L95P, and L139P, respectively). B shows two new mutations found in 5 of 13 patients residing in other geographic areas. Left, the frameshift mutation caused by deletion of the first thymidine in codon 93, resulting in a premature stop codon in exon 4. Right, the splice site mutation (ag→at) at the boundary of intron 2 and exon 3 is shown. The autoradiographs of PCR-SSCP gels show the migration patterns of normal DNA (black arrowheads) and the mobility shifts produced by aberrant alleles (red arrowheads). The sequencing chromatograms below each autoradiograph show the alterations (note the substituted nucleotide marked by an asterisk). In samples that were cloned before sequencing, the normal sequence is absent, whereas in direct sequenced samples, both normal and aberrant sequences are present.*

*SDHD* aberrations, bilateral and multiple PGLs were most common (75%).

Of seven patients with recurrence of a single PGL, four patients harbored a germ-line *SDHD* mutation. We did not detect a germ-line *SDHD* mutation in the patient with metastatic spread in a lymph node.

**SDHD Mutations in Patients Residing in Other Geographic Areas.** To further investigate whether the recurrent mutations could be regarded as founder mutations, we analyzed 13 patients from hospitals in the south of the Netherlands and Belgium for exon 3 mutations. Five (38%) of these patients harbored a germ-line *SDHD* mutation in exon 3, including the Dutch founder

Table 2 Genetic findings and correlations to patient characteristics

Characteristic	SDHD gene alteration				P <sup>a</sup>
	All missense (N = 32)	D92Y (N = 22)	L95P (N = 9)	No alteration (N = 25)	
Patient [no. (%)]					
Sex					0.38
Male (n = 21)	14	10	4	7	
Female (n = 36)	18	12	5	18	
Mean age of onset ± SE (yrs)	40.3 ± 11.4	38.6 ± 9.9	44.8 ± 14.1	44.9 ± 14.2	0.21
Tumor focality [no (%)]					0.0001
Single PGL (n = 30)	8	5	3	22	
Bilateral carotid body (n = 10)	9	7	2	1	
Multiple (n = 17)	15 <sup>b</sup>	10	4	2	
Family history [no. (%)]					<0.0001
Positive (n = 19)	19 <sup>b</sup>	13	5		
Single PGL (n = 2)	2		2		
Bilateral carotid body (n = 7)	7	6	1		
Multiple (n = 10)	10 <sup>b</sup>	7	2		
Negative (n = 38)	13	9	4	25	
Single PGL (n = 28)	6	5	1	22	
Recurrence	4	3	1	3	
Bilateral carotid body (n = 3)	2	1	1	1	
Multiple (n = 7)	5	3	2	2	

<sup>a</sup> The P<sub>s</sub> resulted from  $\chi^2$  tests to compare all variables except mean age of onset, for which we used an unpaired *t* test. All P<sub>s</sub> are for the differences between patients with and without a missense mutation.

<sup>b</sup> Includes the patient harboring the L139P mutation.

mutation D92Y (three patients), a mutation resulting in a change of a splice site (5' intron 2-... *gtat*-3' exon 3), and a frameshift mutation caused by deletion of the first thymidine in codon 93 and resulting in a premature stop codon in exon 4 (Fig. 1B).

## Discussion

PGL is the only well-documented manifestation of the hereditary PGL syndrome, in which *SDHD* gene mutations apparently represent an early and essential pathogenic event (12). We identified *SDHD* mutations in 32 of 57 (56%) unselected patients. This supports the prediction that the *SDHD* gene has an important role in the pathogenesis of parasympathetic PGL. All *SDHD* mutations identified in this study were present in the patients' germ-line DNA, including >30% of patients with a negative family history for PGL. These data indicate that family history is a poor indicator of the risk of hereditary PGL and that screening for germ-line *SDHD* mutations is clinically relevant in all patients presenting with parasympathetic PGLs.

SSCP screening for the presence of mutations revealed four different aberrant patterns. Upon sequencing, three aberrations proved to be missense mutations (D92Y, L95P and L139P), whereas the remaining one was a silent substitution SNP. These mutations could theoretically be clinically unimportant polymorphisms. However, we regard the missense mutations found in this study as pathogenic because they occur at highly conserved residues of the *SDHD* protein, they were absent from 200 control alleles, and finally, the D92Y and L95P mutations have been reported independently as pathogenic in familial PGL (12, 18).

All *SDHD* mutations identified in our study were present in the germ-line DNA; no somatic *SDHD* mutations were found. Recently, one somatic *SDHD* mutation was described in a pheochromocytoma (13). These results imply that the involvement of the *SDHD* gene is restricted to inherited cases of PGL.

It is of interest that only three different *SDHD* mutations were found in the 32 patients with mutated *SDHD*, with two of them, D92Y and L95P, comprising 97% of the mutations detected in this study. This indicates that either these mutations are ancient or that the affected persons are more closely related than known. Information on places of birth in this cohort of patients is suggestive of a founder effect, although some of the patients originate from other European and non-European countries. Baysal *et al.* (12) previously reported the specific D92Y mutation in a Dutch founder population (19). To further investigate whether this founder mutation occurs in patients from other geographic areas, we analyzed 13 patients from hospitals in the south of the Netherlands and Belgium for exon 3 mutations. Aside from the Dutch founder mutation, we also found two new mutations, supporting evidence for founder effects.

The finding of the same germ-line *SDHD* mutations in familial and sporadic cases points most likely to reduced penetrance of the mutated *SDHD* gene, although the occurrence of *de novo* hot spot mutations cannot be completely ruled out. Mutational and haplotype analysis of patients and family members would provide additional information on the origin of the mutations.

In all of the 19 familial PGL patients, a *SDHD* mutation was detected, indicating a high mutation detection sensitivity for SSCP analysis. However, in the apparently sporadic PGL patients, we cannot rule out the possibility that *SDHD* mutations escaped detection by SSCP (20). Furthermore, mutations can be present in the *SDHD* gene outside the screened sequences. It is known that gene mutations in the untranslated regions can severely affect RNA stability. Finally, in some apparently sporadic patients, a germ-line mutation in another gene may cause PGL. Recently, germ-line mutations in the *SDHC* and *SDHB* genes have been demonstrated to cause autosomal dominant PGL syndromes as well (15, 16).

As shown, a positive family history of PGL is the main parameter associated with *SDHD* gene mutations (19 of 19 patients), followed by the presence of bilateral or multiple PGLs (24 of 27 patients). Importantly, in patients without a family history of PGL, bilateral or multiple tumors, but not early age at onset, pointed toward a germ-line *SDHD* gene mutation. In the 28 patients presenting with a single PGL tumor and lacking a family history of PGL, six germ-line *SDHD* mutations were found (21%). No clinical parameter was significantly correlated with any of the three mutations, but the study size precludes the detection of small differences.

These results show that clinical and patient data have limited value in indicating germ-line *SDHD* mutations. Comparable results were reported for apparently sporadic medullary thyroid carcinoma patients, who appeared to have germ-line *RET* gene mutations in about 10% of cases (21). The authors show that, despite this low percentage, the genetic analysis of all apparently sporadic medullary thyroid carcinoma patients is cost-effective.

Genetic counseling on the basis of DNA linkage analysis in PGL families has previously been described by Oosterwijk *et al.* (22). Their results indicate that screening of all PGL patients may further affect counseling strategies because the detection of germ-line *SDHD* mutations in apparently sporadic patients is likely to improve early diagnosis of PGL, which will support adequate and appropriate clinical follow-up and management. On the other hand, we have no data on the lifetime risk of PGLs in people harboring a germ-line *SDHD* mutation. More data are needed to allow a valid estimate of the risk of parasympathetic PGL in patients with *SDHD* mutations. However, our data do support the conclusion that *SDHD* mutation screening should be considered in every patient presenting with this tumor.

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