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RESEARCH ARTICLE

Gross *SDHB* Deletions in Patients with Paraganglioma Detected by Multiplex PCR: A Possible Hot Spot?

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Pheochromocytoma and paraganglioma are rare neuroendocrine tumors that arise in the adrenal medulla and the extra-adrenal paraganglia, respectively. Inheritance of these tumors is mainly a result of mutations affecting the *VHL*, *RET*, *NF1*, and *SDH* genes. Germ-line mutations of the *SDH* genes have been found to account for nearly 10% of apparently sporadic cases. Nevertheless, alterations other than point mutations have not yet been well characterized. In this study, we investigated the frequency of gross *SDH* deletions in 24 patients who tested negative for point mutations and had at least one of the recommended features for genetic testing. For this purpose, we used a technique that is easy to implement in the lab to specifically detect gross deletions affecting *SDHB*, *SDHC*, and *SDHD*. We identified 3 heterozygous *SDHB* deletions (3/24) in 3 independent cases with paraganglioma: 1 whole *SDHB* deletion and 2 deletions exclusively affecting exon 1. These latter mutations match the unique gross deletion previously reported, indicating this region could be a hot spot for gross *SDHB* deletions. It seems likely that these alterations can account for a considerable number of both familial and apparently sporadic paraganglioma cases. Although this is the first report describing the presence of gross deletions in patients with apparently sporadic paragangliomas, the extra-adrenal location of the tumor seems to constitute a determining factor for whether to include these patients in genetic testing for gross deletions in the *SDHB* gene.

INTRODUCTION

Pheochromocytoma (PCC) is a rare catecholamine-secreting tumor derived from chromaffin cells of the adrenal gland. Tumors that arise outside the adrenal gland are usually termed extra-adrenal PCCs or paragangliomas (PGLs). PGLs are highly vascularized and generally benign tumors arising mainly (80% of cases) from neural crest cells of the head and neck or (17% of cases) from intra-abdominal paraganglia of the sympathoadrenal neuroendocrine system (Lack, 1997). PCC occurs in certain genetic disorders such as von Hippel–Lindau disease (VHL), multiple endocrine neoplasia type 2 (MEN 2), neurofibromatosis type 1 (NF 1), and SDH syndromes (Schimke, 1990; Anderson and Lynch, 1993; Maher et al., 1996). On the other hand, hereditary PGLs are mainly associated with germ-line mutations in the succinate dehydrogenase (SDH) subunits: SDHB, the iron sulfur protein, associated with PGL type 4; SDHD, the small integral membrane protein, with PGL type 1; and SDHC, the remaining integral protein of the complex, with PGL type 3 (Baysal et al., 2000; Niemann and Muller, 2000; Astuti et al., 2001).

To date, large phenotype–genotype studies of *SDH* mutations have not been conducted, and little is known about their penetrance and expressivity. Whereas head-and-neck PGLs arise more frequently in *SDHD* mutation carriers, intra-abdominal PGLs and PCCs seem to be associated with *SDHB* mutations (Neumann et al., 2004). Little is also known about the phenotype of *SDHC* mutation carriers, as only a few families have been described to date. However, genetic testing of the 3 genes is usually performed independently of tumor location. In this sense, more recent studies have pointed to the importance of familial antecedents, multiplicity, the presence of extra-adrenal tumors, and young age at onset ($\leq$ 35 years of age)

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TABLE 1. Clinical and Molecular Data of the Patients Analyzed

ID	Age at onset/sex	Tumor	Gross deletion assay			Family history
			SDHD	*SDHB*	*SDHC*	
4	22/F	Multiple abdominal PGLs/PCC	—	—	—	—
11	22/M	Bilateral PCC	—	—	—	—
12	36/F	Bilateral PCC	—	—	—	—
14	38/F	Bilateral PCC	—	—	—	—
18	11/M	PCC	—	—	—	—
21	48/M	Bilateral PCC	—	—	—	—
22	30/M	Restroperitoneal PGL	—	Exon 1	—	—
28	49/M	Bilateral PCC	—	—	—	—
31	14/F	Retroperitoneal PGL	—	Exon 1	—	Father with PGL
32	70/F	Jugulotympanic PGL	—	—	—	—
38	18/M	Carotid PGL	—	Exons 1–8	—	—
41	22/?	PCC	—	—	—	—
47	?/F	PGL of cauda equina	—	—	—	—
48	55/M	Retroperitoneal PGL/PCC	—	—	—	—
57	50/F	Carotid PGL	—	—	—	—
65	38/M	Bilateral PCC	—	—	—	—
71	29/F	Multiple PGLs of head and mediastinal PGL	—	—	—	—
74	14/M	Carotid PGL	—	—	—	—
79	21/F	PGL in Zuckerkandl organ	—	—	—	—
84	40/M	Carotid PGL	—	—	—	—
86	?/F	Multiple abdominal PGLs	—	—	—	—
87	37/F	Jugulotympanic PGL	—	—	—	—
88	28/F	Retroperitoneal PGL	—	—	—	—
98	47/F	Bilateral PCC	—	—	—	Paternal uncle with PCC

ID, identification number; F, female; M, male; PGL, paraganglioma; PCC, pheochromocytoma.

as guidelines for genetic counseling of PGL/PCC patients (Dannenberg et al., 2005).

During the last 5 years, many inactivating point mutations have been described, accounting for both familial and apparently sporadic PGL/PCC cases. Nevertheless, to date only 4 patients with PGL who also carry gross deletions have been reported (Baysal et al., 2004; McWhinney et al., 2004). Three of these patients had familial antecedents; the fourth, who carried a germ-line gross *SDHC* deletion, apparently had sporadic PGL. The scarcity of previous studies and available routine tests makes it difficult to establish the frequency of rearrangements affecting the *SDH* genes. In this sense, it is possible that more cases with *SDH* alterations will be found, further increasing the percentage of germ-line mutations among these patients.

In the present study, we investigated the frequency of gross *SDH* deletions in 24 patients with PCC or PGL who tested negative for point mutations in *RET*, *VHL*, *SDHB*, *SDHC*, and *SDHD* and who had at least one of the recommended features for genetic testing. Because one of the main problems in detecting deletions is the complexity of techniques such as Southern Blotting and semiquantitative real-time PCR, we used a technique that can be easily implemented in the lab.

MATERIALS AND METHODS

Patients

Gross deletion analysis was performed in 24 consecutive patients with PGL and/or PCC (Table 1), aged 14–70, from unrelated families who tested negative for germ-line point mutations in the *VHL*, *RET, SDHB*, *SDHC*, and *SDHD* genes. Genomic DNA was extracted from the patients' blood following a standard method (Sambrook et al., 1989). Informed consent was obtained from all patients.

Multiplex Method

To investigate the presence of deletions affecting the *SDH* genes, we designed a technique based on specific multiplex amplification of each gene. We first designed and labeled (5′6-FAM) a pair of primers for each *SDH* exon. The primer pairs for *SDHB*, *SDHC*, and *SDHD* amplification, designed on the basis of genomic sequences of these genes (Entrez Gene IDs 6390, 6391, and 6392, respectively), were: *SDHB*1F (5′-CCC TTT CTG AGA AGG TCA CG-3′), *SDHB*1R (5′-GGC TTT CCT GAC TTT TCC CT-3′), *SDHB*2F (5′-TTG AAT GCC TGC CTT TTC TAA-3′), *SDHB*2R (5′-AAA CAG AGC CAT CGG ATG AT-3′), *SDHB*3F (5′-ACA TCC AGG TGT CTC CGA TT-3′),

*SDHB*3R (5′-AGC CCA ACA GGA ATG AAA TG-3′), *SDHB*4F (5′-CAG CAA GGA GGA TCC AGA AG-3′), *SDHB*4R (5′-CCC CCA TGC AAA TAA AAA CA-3′), *SDHB*5F (5′-CAG TGT CCA AGA AAT GGG GTA-3′), *SDHB*5R (5′-TGC CAG TTC CTC TCC AGA AT-3′), *SDHB*6F (5′-ATG CAC TGA CCC CAA AGG TA-3′), *SDHB*6R (5′-CCC AGA TTT ACC GAA AGC AA-3′), *SDHB*7F (5′-AGT GAA TTC CCT TTC CTC TGC-3′), *SDHB*7R (5′-TAG GGT TGC TCT CTG CCA AT-3′), *SDHB*8F (5′-TGA ACC AGC TGA GGA AGG AG-3′), *SDHB*8R (5′-TGC TGT ATT CAT GGA AAA CCA A-3′), *SDHC*1F (5′-GTC ACA TGA CAC CCC CAA C-3′), *SDHC*1R (5′-CTC CCA GTC CCA CTG AAG TC-3′), *SDHC*2F (5′-GAA AAT GGT ATC AAG GAC ACT-3′), *SDHC*2R (5′-AGT CCC AGC TAC TCA GGA G-3′), *SDHC*3F (5′-GAT TAC AGG CCT GAG CAA CC-3′), *SDHC*3R (5′-CTG GCT CCA GAA TCC TTC CT-3′), *SDHC*4F (5′-TTC CTT TTT AAA ATT GTC TTT GTG TG-3′), *SDHC*4R (5′-TTC AAA GGA GGC GGA GAC TA-3′), *SDHC*5F (5′-CAG GGG TCC CAG TTT TAT GT-3′), *SDHC*5R (5′-AGA AAA TGT GCA AAT CCC GA-3′), *SDHC*6F (5′-TAA AGG TGG GGC ATA AGG-3′), *SDHC*6R (5′-AGA AAC AAG GGG AAA ACT AGA-3′), *SDHD*1F (5′-ATT GTC GCC TAA GTG GTT CC-3′), *SDHD*1R (5′-CTG GAG GCT ACG CTA AGC AC-3′), *SDHD*2F (5′-TCA GTC CTG TTA AAG GAG AGG TTC-3′), *SDHD*2R (5′-TAG AGC CCA GAA AGC AGC AG-3′), *SDHD*3F (5′- TTT GGG TTA CTG TGT GGC ATA-3′), *SDHD*3R (5′-CAC AGC AAA CAA ACT GAG CA-3′), *SDHD*4F (5′- GTC TTC TAA TTT CAC TGT GGT TTT T-3′), and *SDHD*4R (5′-TTC AAA GTA TGA AGT CAA AAA GGT C-3′). We used control fragments from chromosomes 1, 3, and 11 as internal controls of the assay. To confirm the deletion of *SDHB* exon 1 and to be able to rule out the presence of polymorphisms in the primers' recognition regions, we designed two additional pairs of primers that included exon 1 coding sequences: *SDHB*1AF (5′-AGT GGG TCC TCA GTG GAT GT-3′), *SDHB*1AR (5′-ATC AGC TCC AGG CAG TCT CT-3′), *SDHB*1BF (5′-CTC CGC CCC ATC TAA GTC T-3′), and *SDHB*B1R (5′-TCT CTG AGG CTC CAG GAC TC-3′). We amplified genomic DNA of the patients with a multiplex PCR kit according to standard recommendations (Qiagen GmbH, Hilden, Germany). Briefly, multiplex amplification was performed in 25 μl of a mixture containing 1× multiplex PCR master mix, 0.2 μM of each primer, and 100–200 ng of genomic DNA. The PCR program started with an initial heat-activating step at 95°C for 15 min to activate HotStartTaq DNA polymerase. There were 20 cycles of amplification—30 sec at 94°C, 90 sec at 60°C, and 90 sec at 72°C—followed by a final extension of 10 min at 72°C. PCR amplification products obtained from the *SDH* deletion tests were used for fragment analysis on an ABI Prism™ 310 capillary sequencer (Applied Biosystems, Perkin Elmer, Warrington, UK) and were analyzed using GeneScan v3.1 (Applied Biosystems, Warrington, UK). Normalization was performed on patient samples overlapped with a control sample, which determined the peak surface of all fragments and calculating the normal peak fractions (Schouten et al., 2002).

Quantitative Real-Time PCR

Quantitative real-time PCR assay was carried out using the ABI Prism 7900 Sequence Detection System (Applied Biosystems) and the SyberGreen PCR master mix (Applied Biosystems). DNA content of the samples was measured by picogreen, and both 12.5 and 62.5 ng of DNA were used for amplification in a total volume of 12.5 μl. *SDHB* exon 1 was amplified using *SDHB1F* and *SDHB1R*, and a control gene (on chromosome 3) was quantified for data normalization. The PCR reaction started with 10 min at 95°C; followed by 5 cycles of 20 sec at 95°C, 30 sec at 58°C, and 45 sec at 72°C and 45 cycles of 20 sec at 95°C, 30 sec at 56°C, and 45 sec at 72°C. *SDHB* exon 1 copy number was quantified by measuring the threshold cycle and by using a standard curve to determine the starting copy number (Bieche et al., 2004). The standard curves were developed using serial dilutions of DNA from a control sample without deletions in the selected genes. The 3 samples carrying deletion of *SDHB* exon 1 and 1 additional control sample without a deletion were run in triplicate. The standard deviation of the measurements was calculated for all cases and was always less than 17% of the mean value. The ratio of the copy number of *SDHB* exon 1 to the copy number of the control gene normalized the amount and quality of the genomic DNA. The ratio defining the level of amplification, termed N, is the copy number of *SDHB* exon 1/copy number of control gene. A normal diploid sample was thus expected to yield a ratio of $N = 1$, compared to $N = 0.5$ for a haploid genotype.

RESULTS

We screened 24 patients with PGL and/or PCC for the presence of gross germ-line deletions affecting the *SDHB*, *SDHC*, and *SDHD* genes by

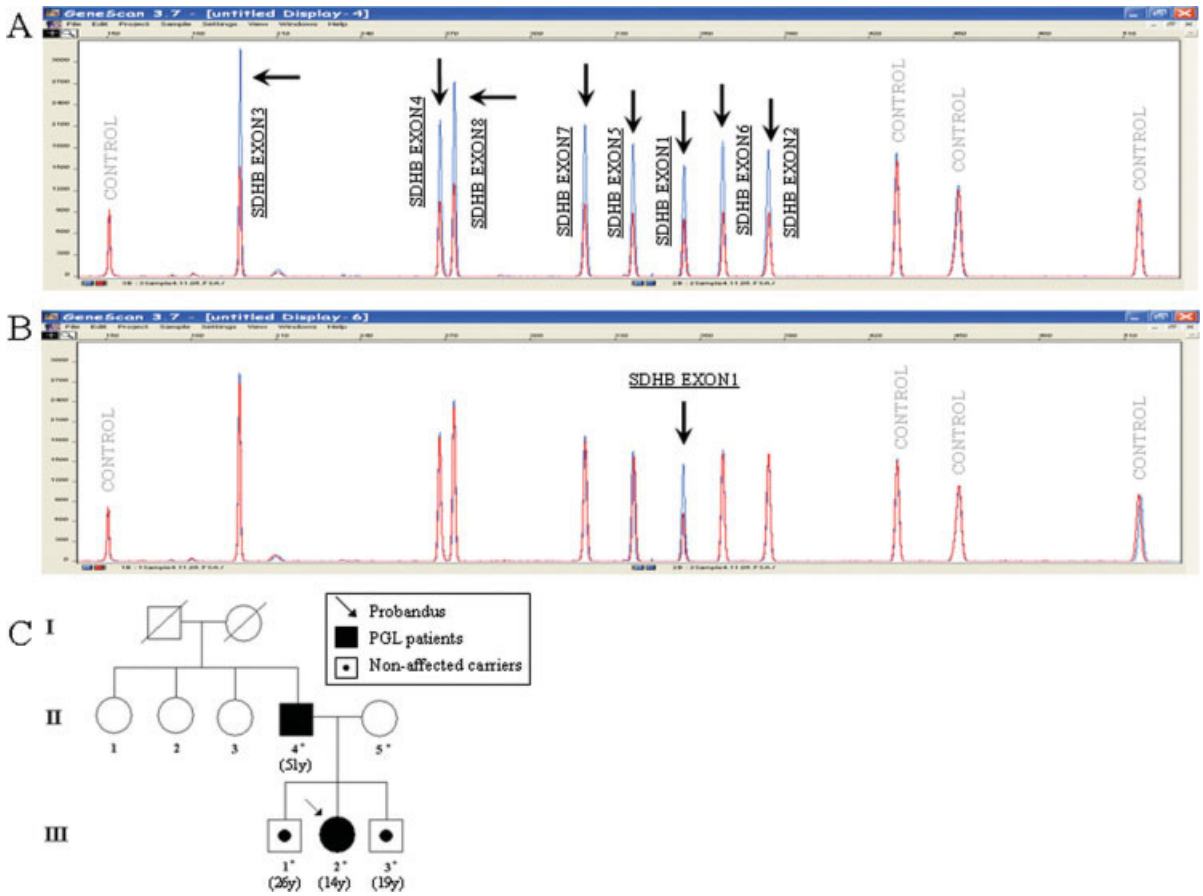

Figure 1. Genescan analysis of amplified PCR fragments using a *SDHB* multiplex assay of a control sample (blue) overlapped with a patient sample (red): (A) case 38, (B) case 31 (fragments showing deletions are marked with arrows); (C) pedigree of case 31, a familial case (*family members tested; a number in brackets indicates the age at onset of an affected individual or the current age of an unaffected carriers).

multiplex amplification followed by quantification of the PCR products. We identified 3 heterozygous *SDHB* deletions (3/24) in 3 independent cases (Table 1): 1 whole deletion of *SDHB* and 2 deletions exclusively affecting exon 1 (Fig. 1).

The deletion affecting the whole *SDHB* gene was found in a 27-year-old male (case 38) with no familial history of the disease. The patient was diagnosed when he was 18 years old and was operated on 8 years later for a secreting PGL of the carotid body. A renal angiolipoma was also detected when he was admitted to the hospital, and his left kidney was surgically resected. Figure 1A shows the heterozygous deletion of all amplified fragments of the *SDHB* gene, compared with the homozygous amplification of the control regions.

Another gross germ-line deletion found affected *SDHB* exon 1 (Fig. 1B) of a 30-year-old male (case 22) with a secreting retroperitoneal PGL and an uncertain family history. The paternal grandmother of the patient died after hepatic metastasis of clear-cell renal cell carcinoma, and a maternal first cousin died after developing a brain tumor. Because we could not obtain blood samples from any relative, we were unable to determine if the alterations were *de novo* or inherited. We confirmed the deletion using two alternative pairs of primers for exon 1 amplification in a new multiplex assay. Amplification and subsequent sequencing using the more external pair of primers allowed us to exclude the presence of polymorphisms in the recognition sequence of the initial *SDHB* exon 1 primers. In addition, we twice confirmed the deletion using quantitative real-time PCR (Table 2).

TABLE 2. *SDHB* Gene Copy Number Quantified Using Real-Time PCR

ID	N	SD
38	0.45	0.00
22	0.43	0.06
31	0.50	0.03
Control	1.06	0.01

N, level of amplification; SD, standard deviation.

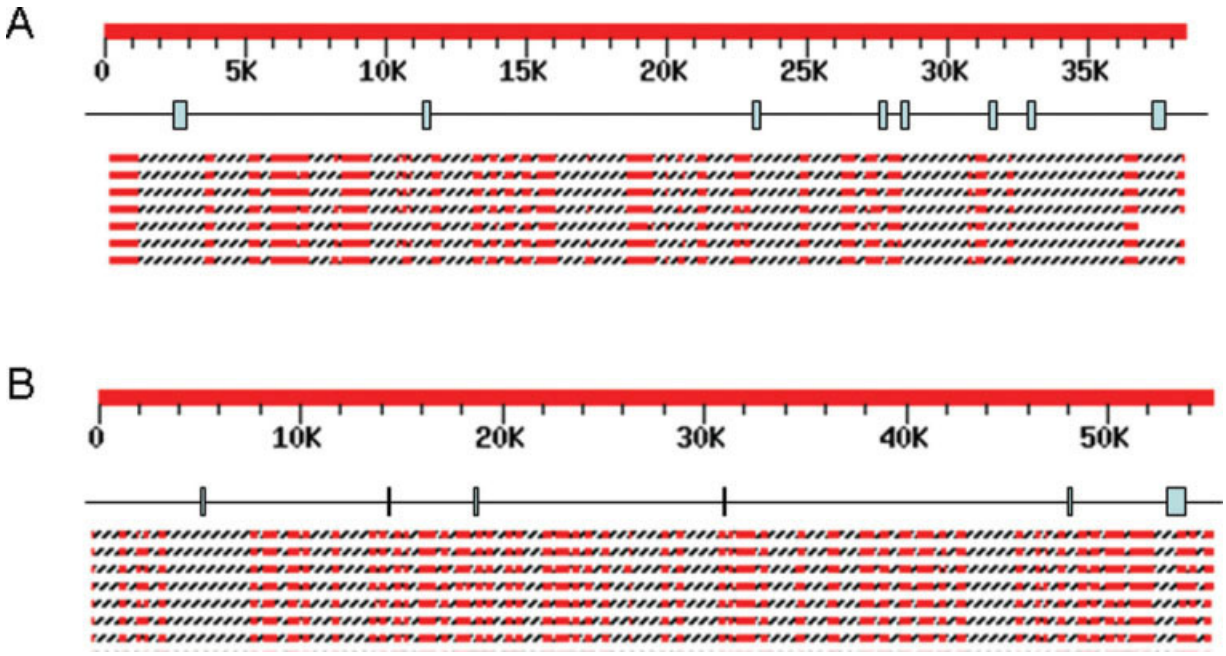

Figure 2. Diagram showing both the genomic structure (exons indicated by boxes and introns indicated by lines) and the distribution of *Alu* repeats of (A) *SDHB* and (B) *SDHC* genes (http://www.ncbi.nlm.nih.gov/BLAST). The *Alu* database sequences aligned to the query sequence are shown in red. Each line under the genomic structure of the genes corresponds to a subfamily of *Alu* repeats with more than 200 scores in the analysis, and each discontinuous line connects multiple alignments on the same database sequence.

The same loss of *SDHB* exon 1 was found in a 14-year-old female (case 31) with familial antecedents of PGL (Fig. 1C). This patient was admitted to the hospital with hypertension and was later diagnosed with catecholamine-secreting PGL of the retroperitoneum. The patient's father was diagnosed at age 48 with PGL of Zuckerkandl's organ, which metastasized to the liver at age 53. In addition, he was admitted to the hospital twice, 2 and 4 years later, with relapse of the hepatic metastasis. During the last admission, bone metastases were also found. The two brothers of the proband also had high levels of dopamine in their urine. We detected the germ-line deletion in all 3 of these relatives of the proband—her father and two brothers. We confirmed the deletion in all carriers by using the two alternative pairs of primers for exon 1 amplification and sequencing and also by quantitative real-time PCR (Table 2).

We did not find any deletions affecting the *SDHC* or *SDHD* genes in any patients analyzed in the present study.

DISCUSSION

Although it has been reported that around 70% of familial PGL cases carry germ-line point mutations in one of the *SDH* genes (McWhinney et al., 2004), it is likely that the percentage of germ-line mutations in these genes is even higher because previously large deletions has rarely been tested for. Regarding the apparently sporadic cases, the main criteria for identifying germ-line mutations in the *SDH* genes are having tumors that are extra-adrenal, bilateral, or malignant, having multiple tumors, and having young age at onset ($\leq$5 years of age).

In the present study, we searched for gross *SDH* germ-line deletions in 24 Spanish patients with at least one of the features of individuals at risk of being mutation carriers and who had previously tested negative for point mutations of the *RET, VHL*, and *SDH* genes. We identified alterations in 3 of 24 cases (12%): 2 patients with deletion of the first exon of *SDHB* and 1 patient who had lost the whole *SDHB* gene (Fig. 1). The deletion of exon 1, which was found in two independent cases, matched the unique gross deletion previously reported by McWhinney et al. (2004), whereas deletion of the entire gene has not been reported before.

No hot spots for point mutations have been described for *SDHB* to date. In contrast, the high frequency of exon 1 deletions (3/4 cases, including the reported case) suggests that this region could be a hot spot for gross *SDHB* deletions. Analysis of the genomic structure of the *SDHB* gene revealed a high density of *Alu* repeats within the first intron (11 elements that covered 36% of the sequence;

Fig. 2). The human genome contains up to 1 million copies of interspersed *Alu* elements (1 *Alu* repeat every 5 kb) that are thought to mediate chromosomal rearrangements and homologous recombination events that result in translocations, duplications, inversions, or deletions (Kolomietz et al., 2002). Although introns 2 and 3 also have a high number of *Alu* elements (16 and 7, respectively), *Alu*-mediated recombination may account for the observed clustering of a gross deletion hot spot. In fact, an *Alu*-mediated *SDHC* deletion was recently described and was found to be the cause of familial and sporadic PGL (Baysal et al., 2004).

One of the patients who carried the partial *SDHB* deletion had familial antecedents of the disease, whereas the other two cases were apparently sporadic. Although the only previous case carrying a germ-line gross *SDHB* deletion was found in a patient with familial antecedents of the disease (McWhinney et al., 2004), a recent report also described an apparently sporadic case carrying a gross *SDHC* deletion (Baysal et al., 2004). In this sense, it seems that the percentage of apparently sporadic PCCs and PGLs carrying germ-line *SDH* mutations could be higher than the previously reported 24% for all 4 genes (Neumann et al., 2002) if the potential gross *SDH* deletion carriers are considered. It is worth mentioning that we did not find any deletion affecting the *SDH* genes in patients with PCC, despite bilaterality or early age of onset. In fact, if only those cases diagnosed with PGL are considered, the percentage of gross deletions found in our study increases to 20%. This is consistent with the recommendations on priorities for genetic testing of *SDH* genes depending on tumor location (Neumann et al., 2004) and also confirms that extra-adrenal PGLs constitute a determining factor for considering these tumors as probable familial cases.

Regarding the age of onset, only the affected father of case 31 was diagnosed after the mean age described for the disease (~30 years old). When this patient was diagnosed, he had clinical symptoms of a neuroendocrine-secreting tumor of several years' evolution. Tumor size and subsequent hepatic metastasis suggested a late diagnosis. This illustrates the malignant potential of *SDHB* tumors (Gimenez-Roqueplo et al., 2003) and underscores the need for early molecular diagnosis of putative *SDHB* mutation carriers. On the other hand, because the penetrance of *SDHB* mutations is 50% at 35 years (Neumann et al., 2004), it was not surprising to find two young unaffected carriers in this family (Fig. 1C).

Among the affected members of the previously reported *SDHB* family (McWhinney et al., 2004), there was 1 patient with an abdominal PGL and 2 individuals with either thoracic or head-and-neck PGL. In the present study, 3 patients presented with abdominal tumors, and only 1 patient had been diagnosed with PGL of the carotid body. This is consistent with a recent report that 50% of adequately followed *SDHB* point mutation carriers developed abdominal extra-adrenal PGLs compared with 21% of *SDHD* carriers (Neumann et al., 2004). Because it appears that the presence of abdominal tumors makes the finding of *SDHB* alterations more likely, screening of gross *SDHB* deletions in patients with extra-adrenal abdominal PGLs who tested negative for *SDH* point mutations could be performed by the following recommended genetic test. Despite the limited number of cases described to date (7, including the 3 found in this study), it seems that the presence of extra-adrenal tumors and early age of onset are the best clues for the early detection of *SDHB* mutation carriers of gross deletions. Nevertheless, taking into account the phenotypic variability related to germ-line *SDH* mutations, alterations in other *SDH* genes cannot be ruled out in cases negative for *SDHB* mutations.

Regarding other tumors related to *SDH* alterations, it has been described that germ-line *SDHB* mutations can predispose to early-onset kidney cancer in addition to paraganglioma (Vanharanta et al., 2004). We had no blood sample available from patient 22's paternal grandmother, who died after hepatic metastasis of clear-cell renal cell carcinoma, so we can neither confirm nor rule out *SDHB* involvement in the development of this pathology. Regarding the renal angiolipoma of case 38, the presence of renal cell carcinoma was excluded by an anatomopathological study.

Appropriate and timely clinical screening of all patients with PGL type 1 and PGL type 4 is recommended (Neumann et al., 2004). In this sense, detection of germ-line alterations is very important in order to correctly follow up on not only affected individuals, but also their relatives at risk of developing the disease. Taking our results into account, it seems likely that gross deletions can account for a considerable number of both familial and apparently sporadic cases (12% in our selected series of cases previously testing negative for point mutations). Detection of these alterations requires complicated techniques that make molecular diagnosis and counseling difficult in many cases (Baysal et al., 2004). We have described a procedure to

detect these alterations that only requires quantification of DNA amplified with a multiplex assay and comparison of the DNA with a normal sample. This protocol is easy to implement in clinical labs and could contribute to uncovering hidden carriers of germ-line alterations.

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