Candidate gene mutation analysis in bilateral adrenal pheochromocytoma and sympathetic paraganglioma

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Abstract

Pheochromocytomas (PCCs) are rare tumors that arise from chromaffin tissue in the adrenal medulla, but can also occur in the abdomen outside the adrenals and are then called sympathetic paragangliomas (sPGLs). According to the literature, between 15 and 25% of apparently sporadic adrenal PCC and sPGL are caused by germline mutations in *RET*, von Hippel–Lindau disease (*VHL*), succinate dehydrogenase subunit B (*SDHB*), or subunit D *SDHD*. However, few studies have addressed the mutation frequency of these candidate genes in selected subgroups of PCC and sPGL, such as bilateral adrenal PCC or extra-adrenal sPGL, and none have looked at somatic mutations by analyzing tumor tissue. Therefore, we have investigated the occurrence of germline and somatic mutations in *RET*, *VHL*, *SDHB*, and *SDHD* in comparatively large series of bilateral adrenal PCC ($n=33$ patients) and sPGL ($n=26$ patients), with the aim of determining the mutation frequency of each of these genes and to establish a genetic testing algorithm. Twenty-one *RET*, two *VHL* germline, and one *SDHD* mutations were found in the patients with bilateral adrenal PCC. In sPGL, one novel *SDHB* germline and one novel *SDHB* somatic mutation were observed. In addition, two *SDHD* germline mutations were found. We conclude that germline *RET* mutations are predominantly found in bilateral PCC, and that somatic and germline *SDHB* and *SDHD* mutations usually occur in sPGL, which has practical consequences for genetic testing algorithms. We suggest that sequential mutation analysis should be directed first at *RET*, followed by *VHL* and *SDHD* for patients with bilateral adrenal PCC at diagnosis, and at *SDHB* and *SDHD* for patients with sPGL.

Endocrine-Related Cancer (2007) 14 453–462

Introduction

Pheochromocytomas (PCC) are rare catecholamine-producing tumors that arise from chromaffin cells of the adrenal medulla, but can also occur outside the adrenal in the abdomen, and are then called sympathetic paragangliomas (sPGL; Lenders *et al.* 2005). Pheochromocytoma-associated syndromes include multiple endocrine neoplasia type 2 (MEN2), von Hippel–Lindau disease (VHL), neurofibromatosis-1, and the familial pheochromocytoma–paraganglioma (PCC–PGL) syndrome (Neumann

et al. 2002). MEN2 is characterized by medullary thyroid carcinoma (MTC) in association with PCC and has three clinical variants, MEN2A, familial medullary thyroid carcinoma (FMTC), and MEN2B (Thakker 2001). The syndrome is caused by germline mutations of the *RET* proto-oncogene, which are mostly (80–96%) found in *RET* exons 10, 11, and 16 (Thakker 2001, Peczkowska & Januszewicz 2005). Somatic *RET* mutations have also been found in sporadic PCC affecting exons 10, 11, and 16 (van der Harst *et al.* 1998b).

The VHL syndrome is an autosomal dominantly inherited tumor syndrome, with a prevalence of 2–3 per 100 000 individuals (Roman 2004). Patients with VHL syndrome have predisposition to develop retinal and central nervous system hemangioblastomas, clear cell renal cell carcinomas, PCC, pancreatic cysts and islet cell tumors, cystadenomas of the epididymis, and endolymphatic sac tumors (Gimm 2005). Both germline and somatic *VHL* mutations have been found in PCC, which include missense, nonsense, splice-site mutations, and small intragenic and large deletions, but most VHL patients with PCC have missense mutations (Neumann et al. 2002, Dannenberg et al. 2003, Gimm 2005).

The PCC–PGL syndrome is caused by mutations in subunits of the mitochondrial complex II, also known as succinate dehydrogenase (SDH), which is involved in the electron transport chain and the Krebs cycle. SDH consists of a flavoprotein (SDHA), an iron–sulfur protein (SDHB), and two anchoring membrane-spanning polypeptides (SDHC and SDHD; Ackrell 2002). Apart from *SDHA*, which is related to a rare neurodevelopmental disorder called Leigh syndrome, all three *SDH* genes have been implicated in the occurrence of PGL. While *SDHC* has only infrequently been described and exclusively in the context of head and neck PGL, *SDHB* and *SDHD* are also associated with abdominal (sympathetic) PGL and adrenal PCC. *SDHB* mutation carriers present predominantly with sPGL, often with a malignant phenotype, although adrenal PCC and head and neck PGL may occur. *SDHD* mutation carriers present more head and neck PGL and, at a lower frequency, adrenal PCC and abdominal sPGL, which are almost always benign (Baysal et al. 2002, Neumann et al. 2004, Amar et al. 2005, Benn et al. 2005, Schiavi et al. 2005).

In VHL disease and the MEN2 syndrome, PCC often have a bilateral adrenal presentation and are occasionally found at extra-adrenal sites (Bryant et al. 2003, Lenders et al. 2005, Machens et al. 2005). In contrast, patients with a germline *SDHB* mutation present with extra-adrenal catecholamine-producing tumors in 50% of cases. Germline *SDHD* mutation carriers also develop extra-adrenal catecholamine-producing tumors (sPGL), and present with bilateral adrenal PCC (Gimenez-Roqueplo et al. 2003, Maier-Woelfle et al. 2004, Neumann et al. 2004, Amar et al. 2005, Benn et al. 2005).

Although knowledge about genotype–phenotype relationships has improved for germline *RET*, *VHL*, *SDHB*, and *SDHD* mutations, only few studies have addressed mutations of these PCC-causing genes in specific series of bilateral PCC and/or sPGL. In addition, there are no studies that have compared

tumor tissue and the corresponding normal tissue for the detection of somatic mutations. To determine the mutation frequency for each of these four candidate genes in a large subset of patients, we have selected 33 patients with bilateral PCC and 26 patients with sPGL, to screen for germline and somatic mutations in *RET* exons 10, 11, 13, 14, 15, and 16, and in all exons of *VHL*, *SDHB*, and *SDHD* genes. In addition, we discuss the significance of *SDHB* and *SDHD* sequence abnormalities.

Materials and methods

Patients

Tissue specimens were retrieved from the archives of the Department of Pathology of the Erasmus MC (Rotterdam, The Netherlands), The University Medical Center St Radboud (Nijmegen, The Netherlands), and the University Hospital Zürich (Zürich, Switzerland) following approval of the experimental design and protocols by the Erasmus MC Medical Ethical Committee. These are all tertiary referral centers for endocrine tumor syndromes. A series of 33 bilateral PCCs and 26 sPGLs was selected for mutation analysis of *RET* exons 10, 11, 13–16 and the entire coding sequence of *VHL*, *SDHB*, and *SDHD*. Of the 33 patients with bilateral PCC, 2 patients had metastases and 31 patients had no metastases. Twenty-six patients with sPGL were selected, of which 15 patients had metastases and 11 patients had no metastases. All samples were coded, so that patient identity was unknown to the investigator. However, a set of clinical data corresponding to the tumor samples was available for further analysis. Throughout this paper, the extra-adrenal catecholamine-producing tumors from the abdomen are designated sPGL. Malignancy was defined as the presence of (distant) metastases at sites where chromaffin tissue is not normally present.

Tissue preparation

Initially, mutation analysis was carried out on tumor DNA. Corresponding normal DNA was used to determine whether a mutation was also present in the germline. DNA was isolated from paraffin-embedded tissues or snap-frozen tissues whenever available, using Puregene (Gentra, Minneapolis, MN, USA), according to manufacturers' instructions. The exclusive presence of tumor tissue was confirmed by making control slides prior to DNA extraction. Positive controls from patients with known mutations and negative controls from normal individuals were included in all experiments.

Denaturing gradient gel electrophoresis (DGGE)

PCR with genomic DNA as template was carried out in a 50 µl mixture of 1 × PCR buffer (Perkin–Elmer Europe, Rotkreuz, Switzerland) containing 10–400 ng template DNA, 200 µM of each intron-based primer (Table 1), and 1 µl *Taq* polymerase (Ampli Taq Gold, Perkin–Elmer Europe). After a hot start of 7 min at 95 °C, a ‘touch-down’ procedure was used consisting of denaturation for 60 s at 95 °C, annealing for 60 s at temperatures decreasing from 60 to 55 °C during the first

11 cycles (with 0.5 °C decremental steps in cycles 2–11), and ending with an extension step for 60 s at 72 °C. Ten cycles with an annealing temperature of 55 °C and 15 cycles with an annealing temperature of 45 °C followed with extension times of 90 s. After a final extension for 10 min at 72 °C, heteroduplex formation was induced by initial denaturation at 98 °C for 10 min followed by incubations at 55 °C for 30 min and 37 °C for 30 min. For DGGE, 10 µl PCR product in 3 µl Ficoll-based loading buffer were loaded onto 10% polyacrylamide gels containing a urea–formamide gradient in 0.5 ×

Table 1 Primers used for mutation analysis

	Forward 5' → 3'	Reverse 5' → 3'	Product size (bp)	Mutation analysis method
<i>RET 10</i>	GCGCCCCAGGAGGCTGAGTG	cgcccgccgcgccccgcgcccgtccgcgcccccg-cgTGGTGGTCCCGGCCGCC	225	DGGE
<i>RET 11</i>	CTCTGCGGTGCCAAGCCTCA	cgcccgccgcgccccgcgcccggcccgccgcgcccc-gccccGAGTAGCTGACCGGGAAGGC	262	DGGE
<i>RET 13</i>	CTCAAGCAGCATCGTCTTTG	AAGGGAGAAAGAGGGAGAAC	328	SSCP
<i>RET 14</i>	GGTTCAAGAGAAAGCTGAGG	CTAGAGTGTGGCATGGTGG	524	SEQ
<i>RET 15</i>	CTCTGCTGGTCACACCAGG	CGGTATCTTTCTAGGCTTC	296	SSCP
<i>RET 16</i>	cgcccgccgcgccccgcgccccgcccccgccccga-aataataaaAGGGATAGGGCCTGGGCTTC	ACCCCAAGAGAGCAACACCC	224	DGGE
<i>VHL 1a</i>	gcgcgAGCGCGTTCATCCTCTAC	cgcccgccgcgccccgcgcccgcgccccgcgcccc-gccccAGGGCCGTACTCTTCGAC	255	DGGE
<i>VHL 1b</i>	gcgcgGCGGAGAACTGGGACGAG	cgcccgccgcgccccgcgcccggcccgccgcgcccc-gccccGTCTCAGACCGTGCTATCGT	418	DGGE
<i>VHL 2</i>	cgcccgccgcgccccgcgccccgccccgcgccccgccccga-aataataaaCTTTAACAACCTTGCTT	cgtcccgGTCTATCCTGTACTTACCAC	266	DGGE
<i>VHL 3</i>	gcgcgTTCCTTGTAAGACCCCTAGT	cgcccgccgcgccccgcgcccgcgccccgcgcccc-gccccAGCTGAGATGAAACAGTGTAAGT	316	DGGE
<i>SDHB 1</i>	GAGCGACCTCGGGGTTAAG	GCTTTCCTGACTTTTCCCTC	157	SSCP
<i>SDHB 2</i>	cgcccgccgcgccccgcgccccgccccgcgccccgccccgT-TTTCCTTTTTGTGAACCTT	AAGCATGTCCCTAAATCAAA	265	DGGE
<i>SDHB 3</i>	cgcccgccgcgccccgcgccccgccccgcgccccgccccG-GAACCTTACATAAATACCACTGGA	cgCTATCAGCTTTGGCCAGC	242	DGGE
<i>SDHB 4</i>	cgcccgccgcgccccgcgccccgccccgcgccccgccccga-aataataaaTGATTCCGGATATGGGTGAG	cgtcccgCCCCCATGCAAATAAAAACA	256	DGGE
<i>SDHB 5</i>	gcgcgTGATGATGGAATCTGATCCT	cgcccgccgcgccccgcgcccggcccgccgcgcccc-gccccgaaataataaaCAGATTGAAACAAT-AAATAGGGA	261	DGGE
<i>SDHB 6</i>	cgcccgccgcgccccgcgccccgccccgcgccccgccccga-aataataaaCCTCTCTTTTCTCCCATAC	cgCAGCAATCTATTGTCTCTTG	252	DGGE
<i>SDHB 7</i>	gcgcgAGCTAATCATCCCTGGTTTT	cgcccgccgcgccccgcgcccggcccgccgcgcccc-gccccgaaataataaaTTGTGAGCACATGC-TACTTC	270	DGGE
<i>SDHB 8</i>	cgcccgccgcgccccgcgccccgccccgcgccccgccccG-TGGGTTTTCCCTTTTCAGTT	cgcccgccgcgccccgcgcccggcccgccgcgcccc-gccccGTGGGTTTTCCCTTTTCAGTT	361	DGGE
<i>SDHD 1</i>	cgcccgccgcgccccgcgccccgccccgcgccccgccccga-aataataaaTGACCTTGAGCCCTCAGGAACG	gcgcgTCAGGGTGGGAAGACCCCT	153	DGGE
<i>SDHD 2</i>	GATCATCCTAATGACTCTTTCC	AGCAGCAGCGATGGAGAGAA	168	SSCP
<i>SDHD 3</i>	cgcccgccgcgccccgcgccccgccccgcgccccgccccG-TTTTATGAATCTGGTCTTTTTG	gcgcgCAACTATATTTGGAATTGCTATAC	245	DGGE
<i>SDHD 4</i>	cgcccgccgcgccccgcgccccgccccgcgccccgccccgT-GATGTTATGATTTTTCTTTTTCT	gcgcgCAATTCTTCAAAGTATGAAGTCA	269	DGGE

Tris–acetate–EDTA (TEA). The amplicons were electrophoresed at 60 °C and 100 V for 16 h. DNA strands were visualized using silver staining as described previously (Komminoth et al. 1994).

Single-strand conformation polymorphism (SSCP) analysis

PCR amplification of tumor DNA was performed with 10–100 ng DNA in a final volume of 15 µl containing 1.5 mM MgCl₂, 10 mM Tris–HCl, 50 mM KCl, 0.02 mM dATP, 0.2 mM dGTP, dTTP and dCTP, 0.8 µCi α³²P-dATP (Amersham), 15 pmol of each forward and reverse primer (Table 1), and 3 U *Taq* polymerase (Ampli Taq Gold, Perkin–Elmer Europe). PCR was performed for 35 cycles at 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, followed by 1 cycle at 72 °C for 10 min. PCR products were electrophoresed overnight at 8 W on a nondenaturing gel, containing 8% polyacrylamide ((49:1) Fluka, Neu-Ulm, Germany) and 10% glycerol (v/v). After electrophoresis, the gel was dried and exposed to an X-ray film.

DNA sequencing

All samples demonstrating aberrant patterns in the DGGE or SSCP analysis were sequenced. PCR was performed in a final volume of 50 µl under identical conditions as the previous PCR, except that this mix contained 0.2 mM dNTPs instead of 0.02 mM dATPs and 0.8 µCi α³²P-dATP. The PCR products were purified using nucleospin Extract II (Macherey–Nagel, Düren, Germany) according to manufacturers' instructions. The purification was followed by a sequence reaction using the Bigdye Terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). To 1 µl purified PCR product, 2 µl Termination Ready Reaction mix, 2 µl of 5× sequencing buffer, 1 µl forward or reverse primer (13.2 pM), and 14 µl deionized water were added. The cycle Sequencing program was performed for 25 cycles at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. This was followed by a precipitation step, adding 13 µl deionized water, 3 µl of 3 M NaAc (pH 5.2), and 64 µl ethanol (100%) to the sequence reaction product and incubating overnight at room temperature before centrifuging for 20 min at 14 000 r.p.m. After washing the samples with 70% ethanol and centrifuging them for 10 min at 14 000 g, the pellets were resuspended in 20 µl formamide (Applied Biosystems, Warrington, UK). Products were analyzed on the ABI Prism 3100 genetic analyzer (Applied Biosystems).

Results

Bilateral adrenal PCC

The series of bilateral adrenal PCC encompassed 31 patients with benign PCC and 2 patients with malignant PCC. The results of the mutation analysis are summarized in Table 2. Twenty-one patients (8 males and 13 females) with germline mutations in the *RET* proto-oncogene were identified in this group (average age 37 years), of which 15 were located in exon 11 (codon 634), 4 in exon 10 (codons 611 and 620), and 2 in exon 16 (codon 918; Fig. 1). No mutations were found in *RET* exons 13, 14, and 15. All of the *RET* mutation carriers had benign PCC. After mutation analyses was performed, it was revealed that 12 patients with *RET* mutations belonged to four families (with 5, 3, 2, and 2 patients; see Table 2). We did not have clinical information about familial occurrence in four of the *RET* mutation-positive cases. The mutation analysis showed two patients with R64P germline mutations in exon 1 of the *VHL* tumor suppressor gene and both had no metastasis. These patients belonged to the same family. In addition, one D92Y mutation was found in exon 3 of the *SDHD* gene. Thus, if we consider each family as a single entity, we would have 14 index patients with bilateral PCC, in whom we found 12 *RET* mutations, 1 *VHL* mutation, and 1 *SDHD* mutation.

None of the patients with bilateral adrenal PCC had mutations in *SDHB*. However, three heterozygous polymorphisms were found in *SDHB* or *SDHD* in two patients harboring a *RET* C634R mutation. These included A6A in exon 1 of *SDHB*, and H50R and S68S in exons 2 and 3 of *SDHD* respectively. On one occasion, *SDHB* A6A and *SDHD* H50R were present in the same patient (Table 3).

Three patients had metachronous appearance of their bilateral PCC. These patients included one with a C634Y mutation who developed PCC with a 3-year interval, and two patients who did not have mutations in *RET*, *VHL*, *SDHB*, or *SDHD*, which developed PCC with a 4- and 6-year interval. Twenty-seven of the patients with bilateral PCC had synchronous PCC.

Sympathetic PGL

The sPGL included 11 benign and 15 malignant tumors. The results are summarized in Table 2. Mutation analysis of *RET* and *VHL* did not show any abnormalities in the 26 patients with sPGL. In contrast, three aberrations were detected in *SDHB* exons 4, 5, and 7. The first aberration revealed a novel mutation, S100F in exon 4, which was not present in normal

Table 2 Summary of results of bilateral pheochromocytoma mutation carriers

Patient	Sex ^a	Age (years)	b/m ^b	Gene	Nucleotide change mutation/polymorphism ^c	Amino acid change polymorphism ^c	Family	Follow-up (years)
F6	m	31	b	<i>VHL</i>	CGC→CCC	R64P	A	21
F28	m	29	b	<i>RET</i>	TGC→CGC	C634R		
				<i>SDHB</i>	CAC→CCC ^c	A6A ^c		
				<i>SDHD</i>	CAC→CGC ^c	H50R ^c	D	26
F37	f	32	b	<i>RET</i>	TGC→CGC	C634R	B	24
F38	m	42	b	<i>RET</i>	TGC→CGC	C634R	B	22
F39	f	42	b	<i>RET</i>	TGC→CGC	C634R	C	24
F41	m	16	b	<i>RET</i>	TGC→CGC	C634R		
				<i>SDHD</i>	AGC→AGT ^c	S68S ^c	D	21
F42	f	29	b	<i>RET</i>	TGC→CGC	C634R	D	24
F65	m	u	b	<i>RET</i>	TGC→CGC	C634R	E	u
F78	f	29	b	<i>RET</i>	TGC→CGC	C620R		2
F79	m	26	b	<i>RET</i>	TGC→CGC	C634R	E	14
F80	f	18	b	<i>RET</i>	TGC→CGC	C634R	B	14
F82	f	24	b	<i>RET</i>	TGC→CGC	C634R	B	u
F84	f	50	b	<i>RET</i>	TGC→CGC	C634R	B	11
F87	m	24	b	<i>VHL</i>	CGC→CCC	R64P	A	11
F89	f	38	b	<i>RET</i>	TGC→CGC	C634R	C	12
F92	f	51	b	<i>RET</i>	TGC→TAC	C611Y	–	12
F102	f	27	b	<i>RET</i>	ATG→ACG	M918T	–	10
F141	m	49	b	<i>RET</i>	TGC→TGG	C634W	–	u
F147	f	27	b	<i>RET</i>	ATG→ACG	M918T	–	u
F165	f	59	b	<i>RET</i>	TGC→TAC	C611Y	–	17
F168	m	72	b	<i>RET</i>	TGC→TAC	C611Y	–	10 [†]
F184	m	53	b	<i>RET</i>	TGC→TAC	C634Y	F	2 [†]
F188	f	25	b	<i>SDHD</i>	GAC→TAC	D92Y	–	38
F194	f	24	b	<i>RET</i>	TGC→TAC	C634Y	F	9

†, Deceased.

^am, male; f, female.^bb, benign; m, malignant; u, unknown.^cNucleotide and amino acid changes representing polymorphisms are given in italics.

DNA of the same patient. The sPGL of this 25-year-old female patient was localized in the bladder. The second variant pattern of *SDHB*, in exon 5, was identified as a heterozygous germline S163P substitution, which is described as a rare polymorphism (Cascon *et al.* 2004). The third *SDHB* aberration represented the heterozygous mutation C243S in exon 7, which was also present in the corresponding normal DNA (Fig. 2).

Mutation analysis of the *SDHD* gene showed four abnormal patterns, of which two represented previously described germline mutations (D92Y and L95P). The *SDHD* D92Y mutation was found in a 52-year-old woman, who had a bilateral adrenal PCC, 27 years earlier, which is in our bilateral series as well (Table 2, patient F188). The L95P mutation was found in a patient with sPGL at multiple abdominal spots. The other two patterns appeared to be the H50R and S68S polymorphisms (Table 3).

There were very few tumors with mutations in this series which prevented us from drawing any conclusions on genotype–phenotype relationships.

Discussion

It has been reported that patients with *RET* or *VHL* germline mutations often present with bilateral adrenal PCC (Bryant *et al.* 2003, Lenders *et al.* 2005), and that part of the germline *SDHB* or *SDHD* carriers develop sPGL (Gimenez-Roqueplo *et al.* 2003, Neumann *et al.* 2004, Dannenberg *et al.* 2005). However, detailed information about the frequencies of these mutations in patients with bilateral adrenal PCC or sPGL is limited. In addition, there have been virtually no studies based on tumor tissue, which allows the detection of somatic mutations in addition to germline mutations. In the present study, we have investigated tumor tissue and the corresponding normal tissue from a unique series of bilateral PCC and sPGL, predominantly from the Netherlands, for germline and somatic mutations in the above-mentioned PCC susceptibility genes. We found 12 *RET*, 1 *VHL*, and 1 *SDHD* germline mutations in the 23 unrelated patients and families with bilateral PCC, and 2 *SDHB* and 2 *SDHD* mutations in the series of 26 sPGLs.

Figure 1 (A) DGGE patterns of healthy control (C), tumor (T), and normal (N) DNA of patients carrying a germline *RET* C634R or C634Y mutation. (B) Sequence results of four of the six germline *RET* mutations detected by DGGE mutation analysis: C611Y, C634R, C634Y, and M918T. (C) Forward and reverse sequence of tumor sample carrying the germline *VHL* R64P mutation found in a patient with bilateral PCC.

The 12 *RET* mutations encompassed three mutations in codon 611 (C611Y), one in codon 620 (C620R), six in codon 634 ($n=4$ C634R, $n=1$ C634Y, $n=1$ C634W), and two in codon 918 (M918T). All of these mutations have been reported previously (Eng 1996, Machens *et al.* 2001, Amar *et al.* 2005). Since other *RET* mutations have been described in exons 13, 14, and 15 (Peczkowska & Januszewicz 2005), we performed additional SSCP mutation analyses for these exons on the PCC which were negative for mutations in *RET* exons 10, 11, or 16 or *VHL*, but no mutations were found. Two patients with bilateral PCC showed the same germline *VHL* mutation (R64P), and because both patients appeared to be related, they were counted as one patient in our series. These cases were previously reported by van der Harst *et al.* (1998a).

The patient with the *SDHD* D92Y germline mutation will be discussed below.

Mutation analysis of the 26 sPGLs showed one novel germline and one novel somatic variant in *SDHB* (C243S and S100F) and two germline mutations in *SDHD* (D92Y and L95P). The novel *SDHB* C243S variant was seen in a patient with bone metastasis. We consider the *SDHB* C243S variant as a pathogenic germline mutation, firstly because loss of the wild-type allele was seen in the sequence analysis results in our study (Fig. 2). Secondly, *SDHB* C243 is highly conserved throughout many species. Thirdly, two studies report patients who developed a malignant PCC and had a *SDHB* mutation affecting amino acid R242 (R242H), which is also highly conserved (Young *et al.* 2002, Neumann *et al.* 2004). The somatic *SDHB* S100F variant appeared to be a mutation

Table 3 Clinical data and mutation analysis results of sympathetic paragangliomas (sPGL)

Patient	Sex ^a	Age (years)	b/m ^b	Gene	Nucleotide change mutation/ polymorphism ^c	Amino acid change polymorphism ^c	Location sPGL	Location metastasis	Follow-up (years)
F4	f	39	m		—	—	Abdomen	Lung, lymph node	4 [†]
F5	f	70	m		—	—	u	u	1 [†]
F7	f	23	m		—	—	Abdomen	Bone	6 [†]
F10	m	41	m		—	—	Bladder	Lymph node	6 [†]
F12	m	25	m	<i>SDHD</i>	CTG→CCG	L95P	Abdomen	Lymph node	11
F13	m	64	m		—	—	Abdomen	Lung, Brain	2 [†]
F15	m	42	m		—	—	Abdomen	Lung	u
F29	f	62	b		—	—	u	—	u
F54	m	56	b	<i>SDHB</i> <i>SDHD</i>	<i>TCT→CCT^c</i> <i>CAC→CGC^c</i>	<i>S163P^c</i> <i>H50R^c</i>	Thorax	—	13 [†]
F81	f	55	b		—	—	Thorax	—	11
F83	m	47	b		—	—	Abdomen	—	u
F88	m	79	b		—	—	Abdomen	—	5 [†]
F122	f	43	b		—	—	Abdomen	—	u
F138	f	70	b		—	—	Abdomen	—	u
F140	u	u	m		—	—	Abdomen	Lung	5
F160	f	25	b	<i>SDHB</i>	TCT→TTT	S100F	Bladder	—	23
F170	f	35	m		—	—	Abdomen	Bone	10 [†]
F185	f	56	b		—	—	Abdomen	—	10
F188	f	52	b	<i>SDHD</i>	GAC→TAC	D92Y	Abdomen	—	38
F207	f	63	m	<i>SDHB</i> <i>SDHD</i>	TGC→AGC <i>AGC→AGT^c</i>	C243S <i>S68S^c</i>	Abdomen	Abdomen, bone	10 [†]
F209	m	30	b		—	—	Abdomen	—	5
F218	u	u	m		—	—	u	Bone	u
F219	u	u	m		—	—	u	Liver	u
F220	u	u	m		—	—	Abdomen	Lymph node	u
F221	u	u	m		—	—	u	u	u
F222	u	u	m		—	—	u	u	u

^am, male; f, female.^bb, benign; m, malignant; u, unknown.^cNucleotide and amino acid changes representing polymorphisms are given in italics. The somatic mutation is depicted in bold.[†]Deceased.

as well, as loss of the wild-type allele was seen (van Nederveen *et al.* 2007). In addition, this amino acid was conserved throughout many species. Mutations in *SDHB* S100 have also previously been described by Pollard *et al.* (2005) in a patient with PGL, and Neumann *et al.* (2004) reported a patient with an sPGL who had a *SDHB* C101Y mutation.

Two polymorphisms were found in *SDHB*: A6A and S163P. The A6A polymorphism was found in one patient (2% of all patients) with an sPGL. A6A was previously reported as a polymorphism with a prevalence of 4% (NCBI: rs2746462), which is in concurrence with our data. The other *SDHB* polymorphism found in our study was S163P (2% of all patients), which was found in a patient with an sPGL. The frequency in our series was in the same range as the 2.3% S163P substitutions found by Cascon *et al.* (2004) in a healthy control population.

Two previously described germline *SDHD* mutations were found in the sPGL group, D92Y and L95P

(Dannenberg *et al.* 2005). Both mutations are known as Dutch founder mutations in head and neck paragangliomas (Taschner *et al.* 2001). The patient with the D92Y mutation also had an adrenal bilateral PCC, 27 years earlier, which were surgically removed and included in our bilateral PCC series. The L95P mutation was found in a patient with a benign PCC, and after 12 years of follow-up, the patient was alive and well. Both patients are previously described by Dannenberg *et al.* (2005).

Two polymorphisms were observed in *SDHD*, H50R in two patients and S68S in one. Since the frequency of the H50R substitution in our series (4% of all patients) is comparable with the frequency that has been reported in the literature for the normal population, we considered it a polymorphism (Perren *et al.* 2002, Cascon *et al.* 2003). The *SDHD* S68S variant was seen in two patients (one with sPGL and one with bilateral PCC) and has previously been described as a polymorphism (Cascon *et al.* 2004).

Figure 2 (A) Altered *SDHB* DGGE pattern in tumor (T1) and corresponding normal (N1) DNA from patient F207 compared with healthy control DNA (C). Note the identical aberrant pattern in both tumor and normal DNA compared with healthy control DNA pattern, indicating germline aberration. (B) Patient F207 tumor DNA sequence result (forward and reverse) of the germline *SDHB* C243S mutation. Note the relative loss of the T nucleotide compared with the A nucleotide (in the forward sequence; arrow), indicating loss of the wild-type allele (with the A nucleotide) in the tumor. (C) Alignment of *SDHB* amino acid sequence (NCBI: NM_003000) of a variety of species. The arrow points to the highly conserved C243.

Interestingly, the *SDHB* and *SDHD* mutation frequencies found in this study are considerably lower (8% for *SDHB* including the somatic mutation and 8% for *SDHD*) than that reported in the study of Amar *et al.* (2005) which were 29% for *SDHB* and 12% for *SDHD*. This difference might be explained on the basis of geographical variation in mutation frequency, as has become evident from comparative studies (Gimenez-Roqueplo *et al.* 2006). Another study reported a mutation range of 30–41% of *SDHB* mutations in a series of catecholamine-producing PGLs (Brouwers *et al.* 2006). This high mutation frequency could be due to a sample bias, as all PGLs were malignant, whereas only approximately half of our sPGLs were malignant. In addition, the technique used in our investigation cannot be used to detect all genetic changes in these candidate genes, i.e. it is not suitable for detecting large deletions, which have been demonstrated in *VHL*, *SDHB*, and *SDHD* (Maranchie *et al.* 2004, McWhinney *et al.* 2004, Cascon *et al.* 2005).

The nucleotide alterations of *SDHB* and *SDHD* discussed above are thought to be polymorphisms (*SDHB* A6A and S163P; *SDHD* H50R and S68S), because they have also been demonstrated in healthy controls. Though, it is striking that out of four patients with either a *SDHB* mutation or polymorphism, three patients also had a polymorphism in *SDHD* (Tables 2

and 3). In our study, one patient with an sPGL harbored the *SDHB* S163P polymorphism and the *SDHD* H50R. In addition, the sPGL with the *SDHB* C243S mutation also showed the *SDHD* S68S polymorphism. To test whether there was a relationship between *SDHB* and *SDHD* polymorphisms and PCC development, we screened an additional series of 89 normal DNA samples of patients with adrenal PCC for the *SDHB* S163P and *SDHD* H50R polymorphisms. However, we did not find additional patients with both S163P and H50R, suggesting that there is no relationship between the polymorphisms and the development of adrenal PCC (data not shown). For sPGL, this could not be assessed, due to the limited number of specimens available. Interestingly, *SDHB* and *SDHD* polymorphisms were recently demonstrated at increased frequency in patients with familial medullary thyroid carcinoma (Montani *et al.* 2005).

In most published series, usually only germline mutations in *SDHB* and *SDHD* have been found, especially since most studies have addressed germline DNA only. In this study, we have chosen to perform mutation analysis on DNA from tumor tissue and the corresponding normal tissue, in order not to miss somatic mutations. We found only a single somatic mutation, in the *SDHB* gene, in our group of 58 patients, although it must be noted that the likelihood of finding such

mutations in bilateral PCC is probably low. In our previous studies, we have shown that somatic mutations in *RET* and *VHL* occur at a low, but not insignificant, frequency. In contrast, somatic *SDHB* and *SDHD* mutations are so far very rare. As such, mutation analysis directed at the detection of somatic mutations in these candidate genes appears not warranted.

In our study, we found 52% *RET*, 4% *VHL*, and 4% *SDHD* germline mutations in 33 patients with bilateral PCC, and 8% *SDHB* and 8% *SDHD* mutations in 26 patients with sPGL. Amar *et al.* (2005) recently described 41% *VHL* ($n=17$), 27% *RET* ($n=11$), and 7% *SDHD* ($n=3$) germline mutations in 41 patients with bilateral adrenal PCC. In addition, these authors found 7% *VHL* ($n=4$), 29% *SDHB* ($n=17$), and 12% *SDHD* ($n=7$) mutations in 58 patients with sPGL. These results are not entirely in concurrence with our data. This occurrence is most likely due to geographical differences, as most of our patients were from the Netherlands, whereas all of the patients of Amar *et al.* (2005) were from France. In addition, geographical differences in mutation frequencies of the PCC susceptibility genes between France, Germany, and Italy have recently been shown by Gimenez-Roqueplo *et al.* (2006). In addition, the technique used in this investigation cannot be used to detect all genetic changes in these candidate genes, i.e. it is not suitable for detecting large deletions, which have been demonstrated in *VHL*, *SDHB*, and *SDHD* (Maranchie *et al.* 2004, McWhinney *et al.* 2004, Cascon *et al.* 2005).

In summary, we have performed mutation analysis on the PCC susceptibility genes *RET*, *VHL*, *SDHB*, and *SDHD* in a series of bilateral adrenal PCC and sPGL. The bilateral adrenal PCC showed only germline *RET*, *VHL*, or *SDHD* mutations and the sPGL only germline and somatic *SDHB* or germline *SDHD* mutations. Our results imply that it is advantageous to first test patients with bilateral PCC for mutations in *RET*, although, based on previous literature, simultaneous testing for *VHL* and *SDHD* appears justified, while patients with sPGL should be first tested for mutations in the *SDHB* and *SDHD* genes. In addition, the finding of rare somatic *SDHB* gene mutations indicates that mutation analysis of tumor DNA should always be considered when germline mutations are not found.

Acknowledgements

The authors declare that there is no conflict of interest of financial or personal kind with respect to the present study. We thank Frank van der Panne for his assistance with the figures.

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