ONLINE MUTATION REPORT

Genetic and epigenetic profile of sporadic pheochromocytomas

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Pheochromocytoma is a neuroendocrine chromaffin staining tumour that usually causes secondary hypertension by oversecretion of catecholamines.[1] Clinically malignant pheochromocytomas are uncommon, although these tumours can metastasise by lymphatic or haematogenous pathways implied in the liver, lymph node, lung, and bone. Ten per cent of pheochromocytomas have been traditionally considered as hereditary tumours and may be associated with von Hippel-Lindau disease, multiple endocrine neoplasia type 2, or neurofibromatosis type 1.[2–4] Recently, the presence of mutations in three (*SDHB*, *SDHC*, and *SDHD*) of the four genes comprising mitochondrial complex II has been associated with the development of the familial forms of these neuroendocrine tumours, either pheochromocytoma or paraganglioma.[5–7] In fact, the distinction between sporadic and familial cases of pheochromocytoma has undergone a great change in recent months since it was reported that almost a quarter of patients with apparently sporadic pheochromocytomas may be carriers of germline mutations of these genes.[8] The authors reported 24% of patients with germline mutations in *VHL*, *RET*, *SDHD*, or *SDHB* genes, thus challenging the traditional train of thought that proposed that only a minority of sporadic cases with mutations in the genes was involved in familial forms of the disease. Moreover, further study[9] also describes the importance of germline mutations in patients with apparently sporadic parasympathetic paraganglioma (PGL).

In this study we have searched for somatic mutations in *SDHB*, *SDHD*, *VHL*, and *RET* in sporadic tumours to investigate the role of these genes in the pathogenesis of sporadic pheochromocytomas. In order to find additional mechanisms involved in the inactivation of these genes, we also performed a study of the methylation status of promoter CpG islands of *VHL*, *SDHB*, and *SDHD* to discover whether this inactivation mechanism affects pheochromocytomas.

Key points

- Pheochromocytoma is a rare, neuroendocrine, chromaffin staining tumour that usually causes secondary hypertension by oversecretion of catecholamines.
- Recent studies have shown that 25% of patients with apparently sporadic tumours had germline mutations in one of the genes related to the disease (*SDHB*, *SDHD*, *VHL*, and *RET*).
- In this study we looked for somatic mutations in these pheochromocytoma related genes in tumours to decipher their role in the pathogenesis of sporadic tumours. We found that 17% of tumours had mutations in the genes studied. All variants were also detected in the normal patient's tissue, with no somatic changes with the exception of one *RET* substitution. When we compared the molecular data obtained through this study with clinical features, no correlation was found.
- Given that our results confirm the absence of somatic mutations affecting these genes, we developed a CpG island methylation analysis of the *SDHB*, *SDHD*, and *VHL* genes in order to find another inactivation mechanism affecting these pheochromocytoma related genes.
- The study revealed an unmethylated promoter CpG island in all cases, suggesting that aside from a necessary search for other mechanisms involved in the possible somatic inactivation of these genes (gross deletions), other genes involved in the sporadic pathogenesis of this type of tumour must also exist.

MATERIALS AND METHODS

Tumoral and normal tissue

Tumoral samples from 35 patients with pheochromocytoma were collected anonymously with no information surrounding the existence of familial antecedents of the disease. This clinical information was subsequently obtained, along with normal tissue from the cases with variants. High molecular weight DNA was extracted from fresh frozen samples following standard procedures[10] and also from paraffin embedded tumours using either the DNA Easy Tissue kit (Qiagen, Chatsworth, CA, USA) or a modification of a previously described protocol.[11] Normal tissue specimens used in the methylation study were collected from autopsy material.

Amplification and sequencing analysis

The analysis was carried out by genomic DNA amplification by PCR and direct sequencing of all eight exons of *SDHB*, all four exons of *SDHD*, all three exons of *VHL*, and exons 10, 11, and 16 of *RET*, using primers and PCR conditions as previously described.[12–15] By using DNA from 150–200 unrelated and unaffected individuals as a control population for single strand conformation polymorphism (SSCP) analysis,[16] the pathogenic or polymorphic character of variants with unknown significance was defined. PCR products displaying mobility shift were subsequently sequenced using an automatic sequencer (ABI PRISM^{TM} 3700; Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Methylation specific polymerase chain reaction

The methylation status of the *SDHB*, *SDHD*, and *VHL* CpG islands was analysed using the methylation specific

Abbreviations: PGL, paraganglioma; MSP, methylation specific polymerase chain reaction; SSCP, single strand conformation polymorphism

polymerase chain reaction (MSP) technique.[17] This assay distinguishes between unmethylated and methylated alleles in a given gene on the basis of sequence changes induced by sodium bisulphite treatment of DNA, which converts all unmethylated, but not methylated cytosines to uracil. Subsequently, the DNA region of interest is amplified with primer pairs specific for methylated versus unmethylated DNA. Normal lymphocytes, and placental DNA that had methylated in vitro with *SSSI* bacterial methylase were used as negative and positive control for methylation, respectively. The MSP primers used are summarised in table 1. In selected samples, the DNA methylation status was confirmed by bisulphite genomic sequencing, as previously described,[18] using the primers summarised in table 1.

RESULTS

We screened 35 pheochromocytomas for the presence of somatic mutations in *VHL*, *RET*, *SDHB* and *SDHD*, using amplification analysis followed by PCR products sequencing. All findings in primary tumours are summarised in table 2.

We identified three *RET* variants in three tumours: p.Cys611Phe in exon 10, p.Cys634Tyr in exon 11, and p.Met918Thr in exon 16. The mutational analysis of normal patient tissue with mutations revealed that p.Cys611Phe and p.Cys634Tyr had a germline origin, while p.Met918Thr resulted from a somatic change. By verifying the clinical data of the p.Cys611Phe and p.Cys634Tyr patients we found antecedents compatible with MEN2A. The first variation, p.Cys611Phe (table 2), was detected in a tumour sample of a female patient who had been diagnosed at 58 years of age with pheochromocytoma and C cell hyperplasia. The p.Cys634Tyr change was present in the primary tumour of a male subject diagnosed at 35 years of age with pheochromocytoma and signs of invasion. The clinical records of this patient revealed familial antecedents of MEN2A syndrome. The patient's sister was 45 years old when she was operated for unilateral pheochromocytoma and she had developed medullary thyroid carcinoma at 44 years of age. The patient's father had bilateral pheochromocytoma and there were three cases of medullary thyroid carcinoma among his grandchildren. Finally, the p.Met918Thr was found in a sporadic tumour of a female patient diagnosed with non-familial pheochromocytoma at 36 years of age.

The analysis of the succinate dehydrogenase genes revealed four new alterations: a missense substitution (p.Ser163Pro) and a 3 bp deletion (p.Ser195del) in exons 5 and 6 of *SDHB* respectively, and two missense substitutions in *SDHD*, p.Glu42Ala and p.Ala90Thr, both in exon 3. Neither of the patients had familial antecedents. Ages of onset, sex, and tumour characteristics are summarised in table 2. All the substitutions were found in constitutional tissue of the patients, supporting their germline origin. Given that neither variant had previously been described, we performed a study in a control population; the p.Ser163Pro variant was found in four individuals of the control population tested by SSCP, while the remaining changes were not found in controls. We also detected six polymorphic variants: g.129586_7insCTTCTT in *SDHB*,[13] p.Gly148Gly, p.Ser68Ser,[5] p.His30His, p.Gly12Ser (19, 20) and g.112759G→A in *SDHD*.[13] Their respective frequencies in the control population are summarised in table 2. There was no relation between the genetic alterations found in this study and the clinical characteristics and behaviour of tumours in terms of invasion, metastatic status, or malignancy.

DNA methylation patterns analysis at the CpG islands of *VHL*, *SDHB*, and *SDHD* did not show any hypermethylation event occurring in the pheochromocytomas studied or in normal adrenal medulla samples (figs 1 and 2).

DISCUSSION

The presence of positive familial antecedents of pheochromocytoma and the presence of bilateral or multiple tumours are the main parameters associated with *RET*, *VHL*, *SDHD*, or *SDHB* germline mutations. Recent studies, however, report that about 20% of sporadic cases without such clinical features are associated with these genes, so the screening of mutations should be considered essential in all patients independent of their family history or the presence of tumour multiplicity and bilaterality. On the other hand, little is known about which genes are involved in the development of sporadic tumours, therefore in this study we firstly intended

Table 1 MSP and bisulphite sequencing primers for the *SDHD*, *SDHB*, and *VHL* promoters

Gene	Type of primer	Primer sequence (5′–3′)	Untreated DNA (5′–3′)	Annealing temperature
MSP				
SDHB	U	TAAATGGGTATGTGTTGTTATTGT AACCACATACAAACAACCAAATAA	CAAATGGGCATGCGCCGCTACTGC AGCCGCGTACGAGCAACCAAGTGG	60°C
	M	TGGGTATGCGTCGTTATTGC CGCGTACGAACAACCAAATAA	TGGGCATGCGCCGCTACTGC CGCGTACGAGCAACCAAGTGG	
SDHD	U	TGATAGTTGTGTTTGTGTATGTGT AAACCACCATCTCATTCCTAAA	CGACAGCTGTGTTTGCGCATGCGC GAACCGCCATCTCGTTCCTGAG	60°C
	M	AGTTGTGTTTGCGTATGCGC AACCGCCATCTCGTTCCTAA	AGCTGTGTTTGCGCATGCGC AACCGCCATCTCGTTCCTGA	
VHL	U	GTTGGAGGATTTTTTTGTGTATGT CCCAAACCAAACACCACAAA	GCTGGAGGATCCTTCTGCGCACGC CCCGGATCCCGCGGCGTCC	60°C
	M	TGGAGGATTTTTTTGCGTACGC GAACCGAACGCCGCGAA	TGGAGGATCCTTCTGCGCACGC CGGATCCCGCGGCGTCC	
Bisulphite				
SDHB		GGAATAAAGAGGTTGAAATYGGG AACCTACATCCACTAAAAACCCAC	GGAATAAAGAGGTTGAAATCGGGG AGCCTACATCCACTGAGGACCCAC	60°C
SDHD	1st set	TGAGGGGAATGGGATGTAGT CTAAAAACTCAAAATCATCCACCAA	TGAGGGGAATGGGATGCAGC CTGAGGGCTCAAGGTCATCCACCAA	60°C
	2nd set	AGGGGATGGAAGTGAGGATTTA AACAAACTCCTAACCCTCTTTC	AGGGGATGGAAGTGAGGACTCA GGCAAGCTCCTAACCCTCTTTC	59°C
VHL	1st set	TAGTGGAAATATAGTAAYGAGTTGG CCCTCCRAAACATTCCCTC	TAGTGGAAATATAGTAACGAGTTGG CCCTCCGAAACATTCCCTC	57°C
	2nd set	TAYGGTGTTGGAGGATTTTTTTG TCTTCTTCAAAACCRTACTCTTC	TACGGTGCTGGAGGATCCTTCTG TCTTCTTCAGGGCCGTACTCTTC	57°C

Y, C+T; R, G+A; U, primers for unmethylated DNA; M, primers for methylated DNA.
Primer sequences are given as forward and reverse for each set.

Table 2 Clinical and molecular data of our panel of pheochromocytomas

ID	*SDHD*	*SDHB*	*RET*	*VHL*	Change	Freq (%)	Age	Sex	Loc/ mult	Size	Mal/ inv	Follow up/met
8	E3				p.Ala90Thr	–	45	F		2×10×1.4		72/no
11	E4				p.Gly148Gly	–	63	M		7		48/yes
13	E3				p.Ser68Ser	2.8	54	F		13×13×8		120/no
18†	E2				p.Glu42Ala	–	76	F		3.7		
19	E2				p.His30His	–	60	F				108/no
26	E3				p.Ser68Ser	2.8	81	F		5×1.5		27/no
27	E1, E3				p.Gly12Ser, p.Ser68Ser	2.5	60	F		3.5		19/no
3*		E5			g.129586_7ins CTTCTT	3.5	35	F		6×4.5×3.5		24/no
17		E5			p.Ser163Pro	2.3	61	M		4.5	Inv	
32‡		E6			p.Ser195del	–	67	F				
33*		E2			g.112759G→A	3.3	19	F			Mal	
2			E10		p.Cys611Phe	–	58	F		12×8×8		42/no
22*			E11		p.Cys634Tyr	–	35	M		2.5	Inv	42/no
31			E16		p.Met918Thr	–	36	F				
1							59	F		11×7.5×6		
4							59	M		4.5×4.5×4.5		12/no
5							32	M		15×12×10		24/no
6							23	F		3.5		48/no
7							63	F		17×14×11		
9							24	M		4.9		
10†								M		6	Mal	Met
12							42	F		5×4.5×3		
14							73	M		9×8		48/no
15							61	M		4.5		
16*							14	F	E-A	6×5×4		36/no
20							38	F				No
21							26	M				No
23							64	M		10×6×5		
24							76	M		4×3×2		
25							23	F		2		13/no
28							41	M		8.3		12/no
29							20	F	E-A/ mut	4×3×2	Inv	49/no
30							56	F		4.8×3.8×1.2		
34							37			2.9×2.5×2.4	Inv	

Affected exon is designed with a E followed by the number of the exon.
SDHB polymorphisms were named according to GenBank accession number: AL049569.
Freq., frequency in control population; Loc/m, location/multicentrity; E-A, paraganglioma extra-adrenal abdominal; Mal/inv, status of malignancy/invasion; met, metastasis.
Follow up is stated in months.
*Family antecedents; †deceased; ‡this patient had suffered three recurrences of the same tumour.

to characterise the mutations of the genes commonly involved in the sporadic form of the disease.

We searched for mutations in *VHL, RET, SDHB*, and *SDHD* in 35 pheochromocytomas (table 2) and we identified six tumours (17%) with pathogenic changes: three mutations of *RET*, one of *SDHB,* and two of *SDHD*. The three mutations found in *RET* (~8.4%) had been described previously.[21–23] The p.Cys611Phe and p.Cys634Tyr changes were found in normal tissue, confirming their germline origin. They affect two conserved amino acids often mutated in MEN2A. The p.Met918Thr variant was somatic and results in the replacement of methionine with threonine within the catalytic core region of the tyrosine kinase domain.[21] This mutation, affecting substrate interactions, has been strongly linked (95%) to MEN2B, and has also been reported in some sporadic pheochromocytomas.[24 25]

One tumour (~2.8%) was found to have an *SDHB* mutation, and was also germline. The change, a 3 bp deletion, eliminates a serine residue of the conserved and functional 4Fe-4S ferredoxin iron−sulphur binding domain of SDHB.[6] The p.Glu42Ala and p.Ala90Thr *SDHD* substitutions (~5.7%) were germline (not previously described) and they did not affect conserved amino acids of the protein. We had no available material to perform further functional experiments, but as they were not detected in controls we considered these variants to be pathogenic changes.

Aside from other substitutions (g.129586_7insCTTCTT, p.Gly12Ser, p.Ser68Ser, g.112759G→A) previously described as polymorphisms,[12 13 20] we found a new variant, p.Ser163Pro. This substitution was found in 2.3% of the control population, so it could be a rare polymorphism.

We found 17% of tumours to have mutations in some of the genes. All the identified changes were also found in the corresponding normal tissue with the exception of one somatic *RET* mutation. We have therefore found 14% of tumours to have germline mutations in some of the genes studied. This result differs from a previous study[8] where 24% of apparently sporadic pheochromocytomas with germline mutations were found in one of the genes related to the development of these neoplasias (*VHL*, *RET*, *SDHB*, *SDHD*), although our study was of both a genetic and epigenetic nature. In addition, no more than one somatic *RET* change in tumours was found, nor were there somatic mutations in either the complex II genes or the *VHL* gene. It has been previously reported that somatic *RET* or *VHL* mutations are rare in sporadic pheochromocytomas,[25] and *SDHD* mutations have been described as rare in neuroendocrine tumours.[26] Our results support these findings, and also confirm the absence of somatic *SDHB* mutations in sporadic pheochromocytomas.[27]

As one of the classical inactivation mechanisms of tumour suppressor genes is the hypermethylation of promoter CpG islands,[28] we attempted to discover whether this mechanism was involved in gene silencing. To date, the methylation status of *SDHB* remains unknown, and this study suggests that hypermethylation of the upstream CpG island of this

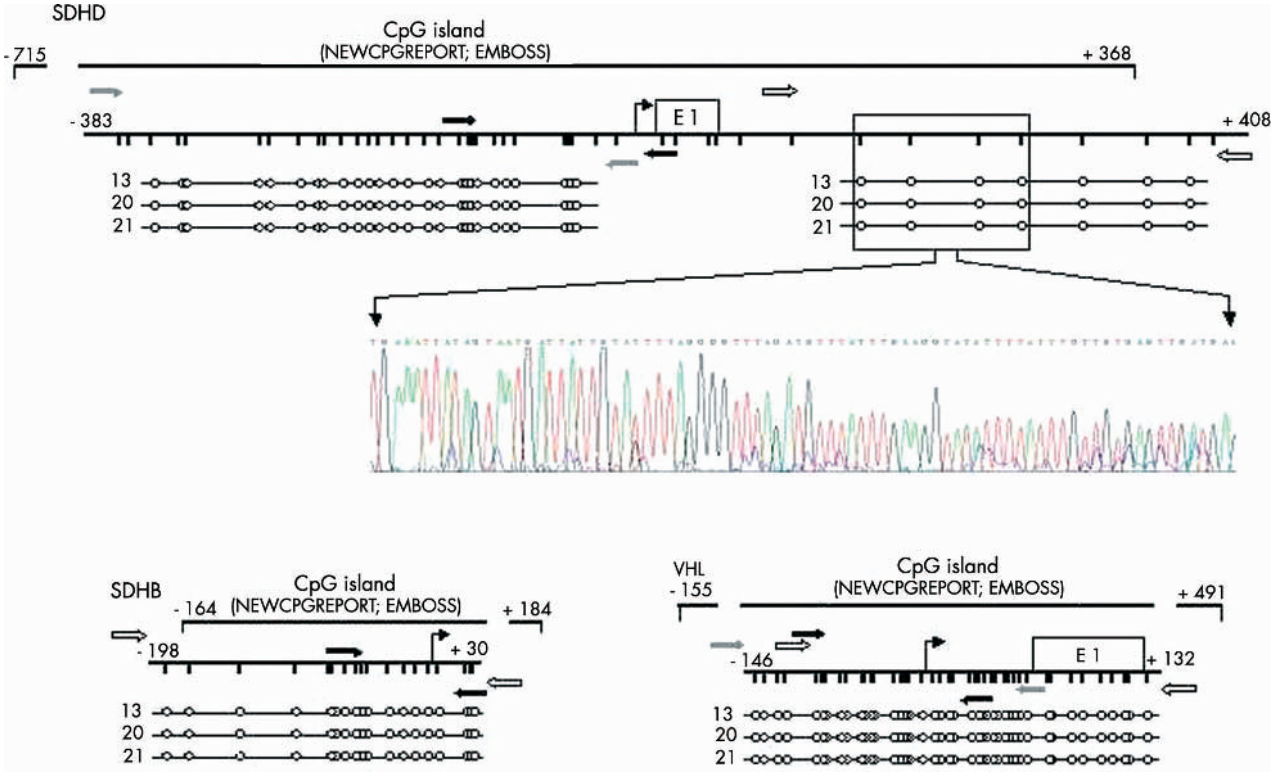

Figure 1 Bisulphite genomic sequencing of *SDHD*, *SDHB*, and *VHL* promoters in several representative human primary pheochromocytomas (13, 20, and 21). The localisation of the CpG island obtained with the NEWCPGREPORT software (EMOSS; http://mammoth.bii.a-star.edu.sg/emboss/index.html) is represented with a horizontal bar. The vertical bars represent the distribution of the CpGs at the *SDHD*, *SDHB*, and *VHL* CpG islands and the vertical arrow indicates the transcriptional start point. Black dots indicate methylated CpGs and white dots unmethylated. The position of the sets of bisulphite sequencing primers used for each gene is represented by grey (for the first set) and white (for the second set) horizontal arrows respectively. The position of the MSP primers is represented by horizontal arrows.

gene is not a frequent event in sporadic pheochromocytomas. On the other hand, it has been reported that *VHL* promoter methylation is not a common event in pheochromocytomas,[29] and in this report we confirm that epigenetic silencing of the *VHL* gene in sporadic pheochromocytomas is not a prominent mechanism, in spite of its relevance in renal cell carcinoma.[30 31]

SDHD, which is believed to be maternally imprinted by means of its inheritance pattern in PGL families, was not methylated at its canonical promoter CpG island in our pheochromocytomas. We also confirmed this finding in three different types of normal adrenal tissue. It has been suggested[5] that it could be an epigenetic modification specifically affecting the carotid body in PGL families, although this event has not yet been molecularly demonstrated. Our results suggest that if methylation related imprinting exists for this gene, it can occur in another region. Moreover, the absence of methylation of this gene in both pheochromocytomas and normal adrenal tissue could indicate that tissue specific methylation is not the underlying mechanism of this pattern of inheritance, and therefore a different molecular mechanism must account for this model.

As no relation was found between the genetic alterations and the clinical characteristics of our series of pheochromocytomas, and given that methylation was not involved in the development of these tumours, other genes may be involved in the pathogenesis of these types of tumours. Further analysis is required in order to identify new candidate genes responsible for tumour pathogenesis of sporadic

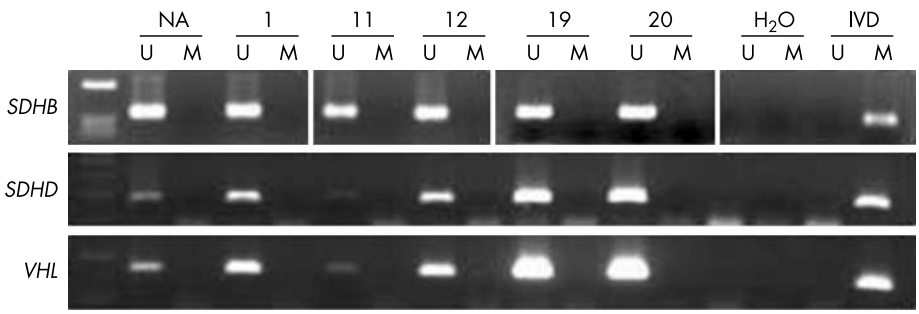

Figure 2 Representative MSP analyses of *SDHD*, *SDHB*, and *VHL* promoters in human primary pheochromocytomas[1 11 12 19 20] revealing promoter unmethylation. The presence of a PCR band under the "U" or "M" lane indicates unmethylated or methylated alleles, respectively. In vitro methylated DNA (IVD) and normal adrenal (NA) DNA were used as positive methylated and unmethylated controls, respectively.

pheochromocytomas and to estimate the hereditary susceptibility to developing this neoplasia.

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