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ARTICLE

Identification of novel *SDHD* mutations in patients with phaeochromocytoma and/or paraganglioma

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Familial paraganglioma is a dominantly inherited disorder characterised by the development of highly vascular tumours in the head and neck. Recently, a relationship between hereditary tumours derived from the autonomic nervous system and germline mutations in the gene encoding succinate dehydrogenase complex subunit D (*SDHD*) is increasingly a subject of study. Familial paraganglioma syndrome is embryologically related to phaeochromocytoma, another neuroendocrine tumour that shows great aetiological and genetic heterogeneity. Some hereditary phaeochromocytomas may be associated with germline mutations in *VHL*, *RET* and *NF1* genes in genetic disorders such as von Hippel–Lindau disease (VHL), multiple endocrine neoplasia type 2 (MEN 2) and neurofibromatosis type 1 (NF 1), respectively. However, there are many cases that cannot be explained by mutations in these genes. In this report, we describe two previously unreported mutations in two patients from 25 unrelated kindreds with phaeochromocytoma and/or paraganglioma disorders and with or without familial antecedents: a mutation featuring the change of tryptophan to a termination codon in exon 2, and a 4-bp deletion in exon 4 that results in a truncated protein. We also describe one missense substitution of uncertain significance. The patients had previously tested negative for germline mutations in *VHL* and *RET* genes and had not been previously selected. The involvement of *SDHD* mutations in familial phaeochromocytoma and/or paraganglioma predisposition is of considerable interest since other studies have shown these alterations to be associated with highly expressed angiogenic factors.
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Introduction

Hereditary paraganglioma is a dominantly inherited disorder characterised by vascular tumours arising in extra-adrenal nonchromaffin tissue. Even though paragangliomas appear in the head and the neck, the most frequent location is the carotid body, the organ that senses oxygen levels in blood.[1] Penetrance of this disease is incomplete when transmitted through mothers since children of affected mothers rarely, if ever, develop the disease, suggesting transmission with maternal imprinting.[2]

Recent analyses of familial paragangliomas revealed some cases with germline mutations in the *SDHD* gene located on chromosome 11q23[2–6] as well as in the *SDHB* gene located on chromosome band 1p35–36.1.[7] The *SDHD* gene is the subject of exhaustive study because it has been postulated that the mitochondrial electron transport chain plays a role in oxygen sensing and signalling.[8] Recent studies have concluded that inactivation of *SDHD* gene in hereditary paraganglioma by means of germline mutations leads to the complete loss of mitochondrial complex II activity

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and stimulates a high level of expression of angiogenic factors, which activate the hypoxia pathway.[9]

Paragangliomas arising from the parasympathetic ganglia share a similar embryological origin with pheochromocytomas.[10,11] Phaeochromocytoma is a neuroendocrine chromaffin-staining tumour that usually causes secondary hypertension by oversecretion of catecholamines.[1] Recent advances in molecular genetics are revealing the nature of the pathogenesis of phaeochromocytoma, with the reporting of multiple genetic alterations. Most phaeochromocytomas are sporadic, but approximately 10% are hereditary and may be found in association with von Hippel–Lindau disease, multiple-endocrine neoplasia type 2 or neurofibromatosis type 1.[11–13] However, the aetiology of most familial and sporadic forms of phaeochromocytoma is still unknown and remains to be characterised.

Since the discovery of germline mutations of *SDHD* in some patients with hereditary paraganglioma,[2] several authors have tried to determine whether this gene plays a role in the development of neuroendocrine tumours.[14] Recently, Gimm *et al*. have found germline *SDHD* mutations in approximately 11% of patients with sporadic phaeochromocytoma, suggesting an involvement of this gene with the disease.[15]

In the present study we have looked for new mutations in the *SDHD* gene and thereby to establish whether the alterations of this suppressor gene are definitely linked to the pathogenesis of tumours derived from the autonomic nervous system and its role in the susceptibility to developing phaeochromocytoma and/or not only familial, but also sporadic, pararaganglioma.

Materials and methods

Patients

The *SDHD* mutation analysis was performed in 25 consecutive patients, without previous selection, with phaeochromocytoma and/or paraganglioma (Table 1), 11 to 68 years of age, with or without familial antecedents and from unrelated families who tested negative for germline mutations in *VHL* and *RET* genes (unpublished data). We used DNA from 140 unrelated and unaffected individuals as a control population. Genomic DNA was extracted from the patients' blood samples following a standard method.[16] Informed consent was obtained from all patients.

Amplification and sequencing analysis

The analysis was carried out by genomic DNA amplification of peripheral blood leukocytes. *SDHD* comprises three introns and four exons. Exon-specific polymerase chain reaction (PCR) and direct sequencing analyses were performed in exons 1 to 4 of the *SDHD* gene. The primer pairs for exon amplification were designed on the basis of the genomic sequences (accession number AB026906) and were as follows: 1F (5′-ATT GTC GCC TAA GTC CTT CC-3′), 1R (5′-CTG GAG GCT ACG CTA AGC AC-3′), 2F (5′-TCA GTC CTG TTA AAG GAG AGG TTC-3′), 2R (5′-TAG

Table 1 Clinical and molecular data of the patients analysed

Patient ID	*Age at onset/sex*	*Paraganglioma*	*Pheochromocytoma*	*Nucleotide change*	*Amino acid change*
1	62/m	–	Unilateral	–	–
2	68/m	–	Unilateral	–	–
3	36/f	–	Unilateral	–	–
4	34/f	–	Unilateral	–	–
5	62/f	–	Unilateral	–	–
6	22/m	–	Bilateral	–	–
7	36/f	–	Bilateral	–	–
8	51/f	–	Unilateral	–	–
9	38/f	–	Bilateral	AGC→AGT	S68S
10	42/f	–	Unilateral	–	–
11	11/m	–	Unilateral	–	–
12	42/f	–	Unilateral	–	–
13	40/m	–	Unilateral	GGT→AGT AGC→AGT	G12S S68S
14	49/m	–	Unilateral	–	–
15	41/m	–	Unilateral	CAC→CGC	H50R
16	37/m	–	Bilateral	–	–
17	14/f*	–	Unilateral	–	–
18	48/m	–	Bilateral	–	–
19	40/m*	Paraaortic and carotid	–	TGG→TGA	W43X
20	22/f	Paraaortic	–	–	–
21	30/m	Abdominal	–	–	–
22	36/f	Head	–	–	–
23	22/f	Paraaortic	Unilateral	–	–
24	20/f	Paraaortic and bilateral carotid	Unilateral	13732delGACT	–
25	32/m*	Paraaortic	Unilateral	–	–

*Patients with familial antecedents

AGC CCA GAA AGC AGC AG-3′), 3F (TTT GGG TTA CAG TGT GGC ATA-3′), 3R (5′-CAC AGC AAA CAA ACT GAG CA-3′), 4F (5′-GTC TTC TAA TTT CAC TGT GGT TTT T-3′), 4R (5′-TTC AAA GTA TGA AGT CAA AAA GGT C-3′). PCR was performed with Gene Amp PCR System 9700 thermocycler (Perkin Elmer, USA) according to the manufacturer's instructions. The purified products were subsequently sequenced using an automatic sequencer ABI PRISM™ 3700 (Applied Biosystems. Perkin Elmer, USA).

Single Strand Conformation Polymorphism (SSCP) and enzyme analysis

SSCP analysis was performed as previously described.[17] PCR products displaying mobility shift were subsequently sequenced. The enzyme analysis was performed by digesting amplified PCR products with 5 u of *Tsp*RI endonuclease and the digestion products were analysed by 3% agarose gel electrophoresis to detect the nucleotide change.

Results

We screened 25 patients with phaeochromocytoma and/or paraganglioma tumours for the presence of germline mutations in *SDHD* gene by amplification analysis followed by sequencing of PCR products. We identified five heterozygous variants (2/7 paragangliomas and 3/18 phaeochromocytomas): one nonsense mutation, two missense substitutions, one silent mutation and one deletion (Table 1).

We found a transition GGT→AGT (G12S) in exon 1 of a 40-year-old male (case 13) with a surgically removed phaeochromocytoma and no family history. This G12S transition resulted in the creation of a Tsp*RI* restriction site found in five of 200 (2.5%) chromosomes of the control population used in the study by means of the enzyme assay designed to screen for this change. The same patient who showed variant G12S had a silent mutation AGC→AGT (S68S) in exon 3. S68S also occurred in a 38-year-old female with bilateral phaeochromocytoma and no family history (case 9).

Another transition, CAC→CGC (H50R), was found in exon 2 of a 42-year-old male (case 15) with an adrenal phaeochromocytoma that had been surgically removed by laparoscopic adrenalectomy (Figure 1). This missense substitution was found in four of the 280 chromosomes tested by SSCP analysis (1.4%). This patient had no family history and tumour samples were not available to perform LOH analysis to confirm the nature of the change.

A 4-bp frameshift deletion in codon 112 (13732delGACT) was detected in exon 4 of a 20-year-old female (case 24). This individual had undergone removal of adrenal phaeochromocytoma and paraaortic paraganglioma, and had bilateral carotid glomus antecedents. No apparent family history was reported for this 20-year-old patient. This unreported frameshift deletion gave rise to a 132-amino acid-truncated protein by creating a premature stop codon.

The second mutation found in this study was a transition, TGG→TGA (W43X), at nucleotide 129 in exon 2

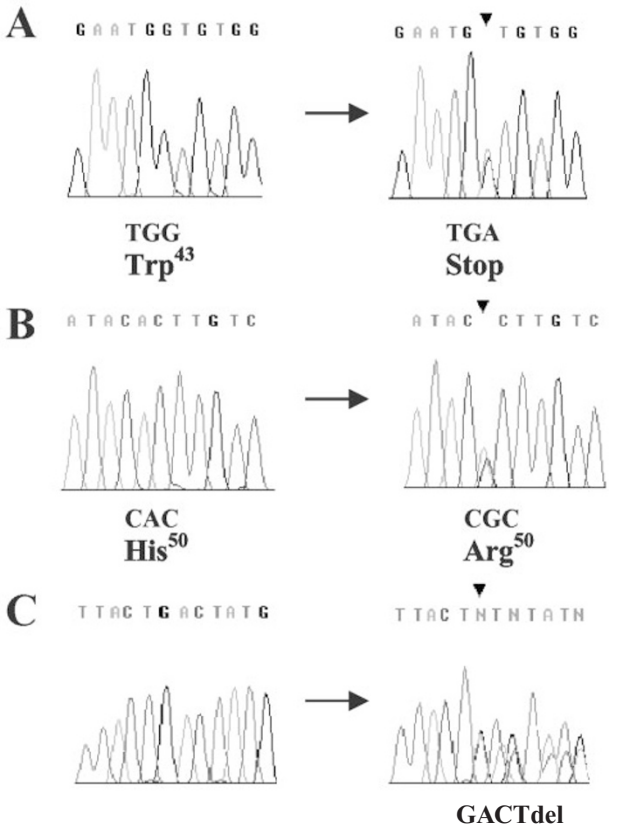

Figure 1 Sequencing chromatograms showing alterations (denoted with one arrowhead). Affected codons and aminoacids are indicated below.

(case 19). This new nonsense mutation of the *SDHD* gene changes a tryptophan to a premature termination codon resulting in a truncated SDHD of 42 aminoacids. The patient with this nonsense mutation was a 40-year-old male when he was admitted to hospital and was the only one with a familial history of phaeochromocytoma. The patient's father died after a secondary hypertensive crisis due to bilateral phaeochromocytoma, and a paternal aunt suffered hypertension and paraganglioma. The patient showed paraaortic paraganglioma and had had two carotid paragaglioma surgically removed.

Discussion

Molecular analysis of the entire coding region of *RET* and *VHL* genes (and even *MEN1*) to find mutations is the commonest method used in paraganglioma and phaeochromocytoma diagnosis, but currently the alterations found in these genes alone cannot explain the development of such tumours.

Germline mutations in *SDHD* have been recently reported as causing hereditary and nonfamilial paraganglioma, implying a tumour suppressor role for SDHD.[18] Linkage between *SDHD* mutations and familial phaeochromocyto-

ma also seems to be a possible mechanism for tumour susceptibility, and so inactivating mutations of the *SDHD* gene appear to be of considerable interest in individuals with familial, multiple, or early-onset phaeochromocytomas and even in nonfamilial phaeochromocytoma.[15,19] However, to date there are few available data on this matter and this aspect needs further evaluation.

SDHD maps to chromosome band 11q23 and encodes succinate dehydrogenase complex subunit D, the small subunit of cytochrome *b* in complex II (succinate-ubiquinone oxidoreductase).[20] Complex II shares important funtions in both the tricarboxylic acid cycle and the aerobic electron transport chain of mitochondria and constitutes the only direct link between activity in the citric acid cycle and electron transport in the membrane.[21] *SDHD* seems to be essential for the interaction between the complex and quinone species.[22]

So, the inactivation of *SDHD* gene in hereditary paraganglioma leads to a complete loss of mitochondrial complex II activity.[9] This mechanism disrupts the hypoxia pathway by means of chronic hypoxic stimulation and cellular proliferation and could be responsible for some cases of paraganglioma and phaeochromocytoma tumours.

In this study, we searched for mutations in the *SDHD* gene in 25 patients with phaeochromocytoma (18 cases), paraganglioma (four cases) alone, and with both (four cases). The patients, with or without familial antecedents and without previous selection, were previously analysed for VHL and RET mutations. Among the samples examined we identified five germline *SDHD* variants distributed among the four exons of the gene: G12S, S68S, H50R, W43X and 13732delGACT. Gimm *et al*. found G12S change in approximately 1.3% of their control population, and they postulated that this variant was either a pathogenic and low-penetrance mutation or a very rare polymorphism.[15] G12S variant changes not only the glycine residue of the sequence GALGGR of SDHD protein but also, therefore, a putative sequence of N-myristoylation that could be important in proteolytic processing.[23] However, the presence of this change in 2.5% of the control population led us to conclude that this change constitutes a polymorphism.

The S68S variant was also found in all the five controls that tested positive for G12S change and in a patient with spinal paraganglioma,[4] so we can conclude that these variants are in linkage disequilibrium.

The H50R missense substitution found in exon 2 causes an amino acid change that could alter the protein conformation. Despite both aminoacids being hydrophilically charged, arginine is more positively charged than histidine under neutral conditions.[24] Histidine 50 is located in a zone of the potential transit peptide of the protein that is conserved in human, bovine and murine SDHD. LOH and segregation analyses were not carried out since neither tumour samples of patients or relative's samples were available. The SSCP analysis revealed the arginine variant in four of 280 control chromosomes (1.4%) so we can postulate either that this is a change of uncertain significance or a rare polymorphism. Further studies will be necessary to clarify the importance of this substitution.

The two mutations, 13732delGACT and W43X, found in exons 2 and 4 respectively, both yield a truncated protein. The 4-bp frameshift deletion caused a truncated protein of 132 aminoacids by creating a premature stop codon, which lacked part of the second transmembrane domain of the protein. This mutation appeared in a 20-year-old female with several tumours derived from the sympathetic nervous system. The change was found in a suggested mutational hot spot[4] located within the same five nucleotides where three different alterations (1-bp insertion, a transition and our 4-bp deletion) have been found.

The W43X nonsense mutation created a premature termination codon resulting in a truncated SDHD of 42 aminoacids that lacked the transmembrane, signal and heme-binding domains. This new germline mutation affected a 40-year-old patient with paraaortic paraganglioma and two operated carotid paraganglioma and a familial history of phaeochromocytoma. Gimenez-Roqueplo *et al*. described a nonsense mutation (R22X) featuring a complete loss of complex II enzymatic activity in inherited phaeochromocytoma not detected in sporadic phaeochromocytomas.[9] We can assume that the same loss has occurred in the W43X mutation.

The identification of new mutations in genes like *SDHD* could represent a new approach to the determination of paraganglioma and phaeochromocytoma susceptibility. Presymptomatic diagnosis of at risk individuals can be carried out by molecular analysis of this gene, and perhaps these novel *SDHD* mutations could help us determine whether the relationship between the respiratory chain and oxygen sensing may be considered to be a mechanism for tumour susceptibility.

In summary, despite it having been reported that complex II deficiency is a rare condition in humans,[25] we have described two mutations in *SDHD* (W43X, 13732delGACT) that resulted in complex II inactivity in 2/7 patients with paraganglioma, one of them also with pheochromocytoma. We have also found one missense variant of uncertain significance and two polymorphisms in linkage desequilibrium in patients with phaeochromocytoma.

The results obtained in this study raise the possibility of applying this approach as a routine genetic screening in patients who test negative for germline mutations in other genes associated pathologies such as *RET* or *VHL*.

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