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Malignant phenotype and two *SDHD* mutations in a family with paraganglioma syndrome type 1

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Summary

Background: Paraganglioma syndrome type 1 (PGL1) is a rare autosomal dominant syndrome associated with multiple, overwhelmingly benign, pheochromocytomas and paragangliomas, attributed to *SDHD* gene mutations. Objective: Clinically and molecularly characterize a family with uncommon malignant phenotype of paragangliomas attributed to two seemingly pathogenic *SDHD* germline mutations. Materials & methods: The proband presented with large bilateral carotid body tumours and family history of cervical masses in his five siblings. All family members underwent clinical examination, imaging studies (^{18}F-FDG PET/CT) and genotyping of relevant genes. The proband was diagnosed with locally advanced paraganglioma; his hypertensive, otherwise asymptomatic father, had locally advanced pheochromocytoma and his three siblings showed multiple head and neck masses, confirmed to be paragangliomas with local metastasis. All affected patients carried two germline mutations in the *SDHD* gene; a previously reported nonsense mutation in exon 1 (p.Trp5X) and a novel missense mutation in exon 2 (p.Pro53Leu), highly deleterious by *in silico* analysis. Allelic loss at the *SDHD* locus was not shown for any of the analysed tumours. Conclusions: This is a rare case of malignant PGL1 with seemingly double pathogenic mutations in the *SDHD* gene, highlighting the possibility that the presence of both mutations is associated with the more aggressive phenotype.

1. Introduction

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are rare, mostly benign neuroendocrine tumours that arise in the adrenal medulla or in extra-adrenal paraganglia, respectively (Dahia, 2014). While the majority of PCCs and sympathetic PGLs are clinically functional and secrete catecholamines leading to distinct set symptoms, the majority of parasympathetic PGLs are asymptomatic but can exert a local mass effect. About 30% of all PCCs and PGLs are caused by germline mutations, especially if associated with hereditary tumour syndromes, such as multiple endocrine neoplasia type 2 (MEN2, MIM #171400), von Hippel-Lindau (VHL, MIM #193300) and PGLs syndromes types 1 to 4 (MIM #168000, MIM #601650, MIM #605373 and MIM #115310) (Kantorovich *et al.*, 2010; Welander *et al.*, 2013). PGLs are dominantly inherited disorders caused by mutations in the succinate dehydrogenase (SDH) complex, encoded by *SDHA, SDHAF2, SDHB, SDHC* and *SDHD* genes. Specifically, paraganglioma syndrome type 1 (PGL1) is attributed to germline mutations in the *SDHD* gene and is clinically manifested as multifocal head and neck tumours with or without benign sympathetic PGLs and PCCs, and a parent-of-origin effect: with maternally transmitted mutations, phenotypic expression is unlikely, though this has occasionally been reported (Neumann & Erlic, 2008; Pigny *et al.*, 2008; Yeap *et al.*, 2011) whereas a near complete penetrance is reported for paternally inherited mutations (Pasini & Stratakis, 2009). In all but one (Amar *et al.*, 2005) of the cases

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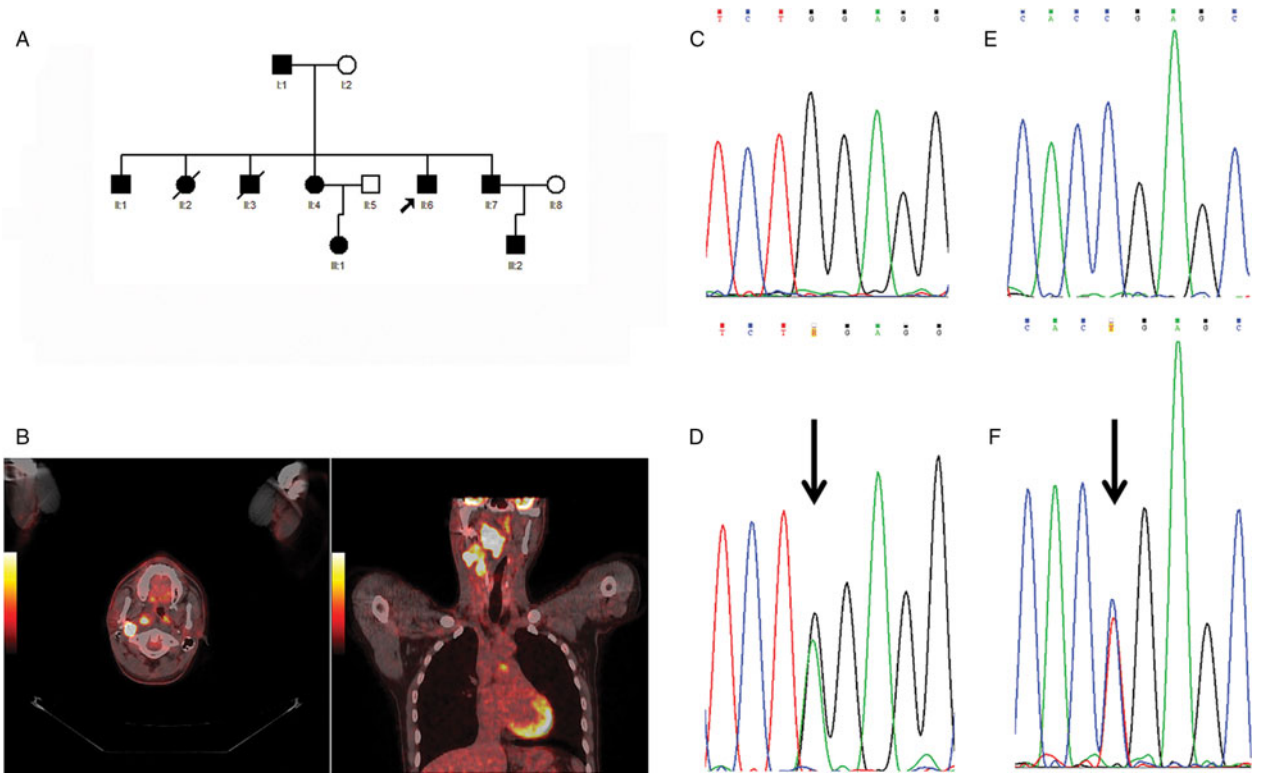

Fig. 1. (*a*) Pedigree shows genotype of family members. Arrow denotes proband; solid symbols, individuals with both mutations (p.Trp5X and p.Pro53Leu), open symbol denotes the mother, wild-type for both mutations; (*b*) Proband (II:6), showing large bilateral masses at the topography of the carotid bodies with several local metastasis. A large tumour at the aorticopulmonary ganglion (aorticopulmonary paraganglioma) is also present; (*c*) sequence analysis of DNA from I:2; (*d*) sequence analysis of the proband's DNA showing a heterozygous nonsense mutation c.14G>A (p.Trp5X) in exon 1; (*e*) sequence analysis of DNA from I:2; (*f*) sequence analysis of the proband's DNA showing a heterozygous nonsense mutation c.158C>T (p.Pro53Leu) in exon 2.

described thus far, a single pathogenic mutation has been detected in affected family members.

In the present study, a family carrying two seemingly pathogenic mutations in the *SDHD* gene presenting with a malignant PGL1 phenotype is described.

2. Materials and methods

(i) *Ethics statement*

The study was approved by the Ethics Committee of UFMG and all participants (pedigree shown in Fig. 1(*a*)) signed an informed consent.

(ii) *Clinical case report*

The proband (II:6), a 38 year old Brazilian male, complained of slow growing, painful, bilateral cervical masses over the preceding 12 years associated with mild dysphagia over the past 24 months. Surgical resection of the right neck mass revealed PGL of the carotid body with affected local lymph nodes. Only after this risky surgical approach and ensuing biopsy results was the patient referred to our Hospital where image study by ^{18}F-FDG-PET/CT (GE Discovery PET/CT 690, GE Healthcare, Milwaukee, USA) revealed bilateral masses at the topography of the carotid bodies with several regional metastasis. A large tumour at the aorticopulmonary ganglion (aorticopulmonary PGL) was also present (Fig. 1(*b*)). During this hospitalization the patient was normotensive and biochemical tests demonstrated that no catecholamine hypersecretion were elicited as urine and blood catecholamine levels were within normal values even in the presence of other masses. His father (I.1) was hypertensive but otherwise asymptomatic. Diagnostic workup of the father, including ^{18}F-FDG-PET/CT revealed a left adrenal gland mass with local metastasis with serum and urine catecholamine levels within normal range. Due to the father's age (84 years) and his general health, no surgical procedure was offered and he remains under tight clinical surveillance.

The family history is also noted for a 42 year old sister (II:4) with bilateral cervical tumours pathologically diagnosed as PGLs who had undergone surgery 2 years prior to the current study. Recent ^{18}F-FDG-PET/CT showed multiple cervical lymph nodes consistent with local metastasis (data not

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shown). Her sibling (II:7) presented with large bilateral tumours of the carotid bodies, with the tumour on the right side causing mass effect and displacing the oesophagus, with locally affected lymph nodes. The PET/CT also showed an additional tumour in the aortic pulmonary ganglion, which is thought to be another primary PGL.

Noteworthy, two additional siblings died about 20 years before the proband came to medical attention: a sister (II:2) died at age 32 years of eclampsia during her first pregnancy, and a brother (II:3) who died at age 25 years of sudden death. No autopsies were performed and no additional data on these siblings is available.

There were no syndromic features of any other known related syndromes, such as MEN2, neurofibromatosis type 1 and VHL; there were no neurofibromas, *café au lait* macules, other endocrine-related tumours or elevated calcitonin levels in any of the tested family members.

Both parents and the two brothers were hypertensive and the genotyped grandchildren were clinically unaffected. Only the younger brother (II:7) who did not undergo surgery prior to this study agreed to take additional biochemical tests. His biochemical profile revealed increased plasma norepinephrine (520 pg/ml) and normal urinary levels of metanephrines, normetanephrines and vanillylmandelic acid.

(iii) *DNA extraction and mutational analyses*

Peripheral blood was collected from all eight family members (Fig. 1(*a*)) in EDTA containing tubes and genomic DNA isolated using the high salt method (Lahiri & Nurnberger, 1991). DNA from fresh tumour of the proband (II:6) and from formalin-fixed paraffin-embedded microdissected tissue of an affected sister (II:4) was isolated according to a proteinase K-based standard protocol (Miller *et al*., 1988).

PCR was performed using primers for the four exons of the *SDHD* gene and eight exons of the *SDHB* gene (primer sequences available on request). Purified PCR products were sequenced with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA) and analysed using Sequencher 4·9 software (http://genecodes.com; accessed 18/9/2014). Each mutation was confirmed by sequencing two independent samples/PCRs. All nucleotide numbers refer to the wild-type genomic DNA sequence of the *SDHD* gene as logged in NCBI (ENST00000367975; NM_003002·3). *In silico* prediction of mutation effect on protein function was performed using Poly-Phen-2 (http://genetics.bwh.harvard.edu/pph2/; accessed 18/9/2014), Mutation Taster (www.mutationtaster.org) and Sort Intolerant from Tolerant (SIFT) (http://sift.jcvi.org; http://sift.jcvi.org; accessed 18/9/2014).

3. Results

(i) *Mutational analyses*

Based on the presence of a malignant phenotype in the studied family and the fact that malignant disease is most commonly encountered in PGL4, a disorder associated with germline *SDHB* mutations (Lenders *et al*., 2014), all coding exons of the *SDHB* gene were initially sequenced in the proband, but no mutations were detected (data not shown). Subsequently, the four coding exons of the *SDHD* gene were sequenced and revealed a previously reported (Neumann *et al*., 2004) heterozygous, stop-codon mutation in exon 1 – c.14G>A (p. Trp5X) (Fig. 1(*d*)). An additional heterozygous missense mutation was also detected in the same patient – c.158C>T, p. Pro53Leu (Fig. 1(*f*)). Co-segregation analysis showed that the father, all four affected siblings and two asymptomatic offspring carry both mutations (data not shown). The mother didn't carry these mutations and was not clinically affected, which was confirmed by ^{18}F-FDG-PET/CT. DNA from 50 healthy individuals randomly recruited from the outpatient clinic in the same medical center during the same time period was sequenced for the two mutations in the gene and none carried either mutation. Score prediction of the missense mutation (p.Pro53Leu) using PolyPhen2 was 0·998, SIFT score was 0·01 and it was deleterious using Mutation Taster.

Analysis of tumours from two affected individuals (II:4 and II:6) showed that both tumours harboured both mutations in a heterozygous mode. Thus, no allelic loss was demonstrated for these tumours at the SDHD locus. Repeated testing with careful microdissected tumour tissue and subsequent analysis of carefully dissected fresh frozen tumour (from patients II:4 and II:6) also failed to show allelic loss.

4. Discussion

The current study highlights two unique features in PGL syndrome. It is the first description of two seemingly pathogenic mutations in the same gene – *SDHD* – that co-segregate with the phenotype across three generations. The p.Trp5X truncating mutation, a previously described (Neumann *et al*., 2004) mutation that is clearly pathogenic, is predicted to lead to a truncated, inactivated protein. Additional evidence to its pathogenicity is the fact that neither Neumann *et al.* (2004) nor we could detect this sequence variant in 600 and 50 healthy control individuals, respectively. The presumed pathogenicity of the p.Pro53Leu missense mutation is less obvious. Yet, even though three prediction algorithms (PolyPhen, SIFT and Mutation Taster) scored this mutation as possibly damaging and deleterious, it has not been reported as a polymorphism in the

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1000 Genomes data base and was not detected in 50 Brazilian controls. These indirect lines of evidence support the notion of pathogenicity, but do not conclusively prove it.

Both mutations are probably located in the same allele, since they are being co-transmitted over three generations. A formal proof of this would be impossible in this family as there are no living affected individuals with a single mutation and the rest of the family seems unwilling to cooperate in order to facilitate comprehensive allelotyping. One can only speculate as to the clinical significance of the coexistence of these two *SDHD* mutations. It may account for the malignant phenotype, namely the missense mutation may be associated with penetrance and expressivity of the mutant truncating mutation carrying allele thereby acting as a modifier of the actual causal mutation. Furthermore, the patients reported by Neumann *et al.* (2002) and Badenhop *et al.* (2004) with the Trp5X mutation presented no metastases, suggesting a more benign event. Yet, it should be emphasized that this biological account is a mere speculation and a more likely explanation is that the missense mutation is simply a rare polymorphism.

Another unique feature in this family is the fact that the disease displayed a malignant phenotype, albeit with a slow growing pattern. Although well described in PGLs associated with *SDHD* gene mutations, it seems to be a rare feature of PGL1, with rates below 4% (Martins & Bugalho, 2014). Moreover, there was a paucity of symptoms in some members of the family: the father is modestly symptomatic (hypertensive) while others in the same family (his offspring II:1, II:4, II:6 and II:7) and possibly two additional offspring who died at an early age (II:1 and II:2) seemingly have symptomatic disease. This may reflect the effect of modifier factors – genetic or environmental. Alternatively, it may imply, at least in this family, that there may be anticipation in the phenotype or it may just be a coincidence. Notably, these are all speculations.

SDHx genes are believed to function as classical tumour suppressors, displaying loss of heterozygosity (LOH) of the non-mutated allele (Welander *et al.*, 2011). However, we could not show LOH of any tumour analysed and both mutations were heterozygous in all tumour samples genotyped. Given the meticulous dissection of the tumour samples, in order to ensure a high rate of enrichment for tumour cells in the analysed samples, the possibility that there is a major contamination of non-tumour cells in the analysed sample seems unlikely. Another possibility is that other mechanisms (e.g. methylation of the wild-type allele) act to inactivate the non-mutant allele, similar to what has been shown in other cancer types (Esteller *et al.*, 2001).

In conclusion, a phenotypically and genetically unique family with aggressive, locally advanced PGLs with seemingly double pathogenic mutations in the *SDHD* gene is described. Careful analysis of all family members of PGL families should be performed regardless of age and symptoms.

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Declaration of interest

None.

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