

ONLINE MUTATION REPORT

Phenotype variability of neural crest derived tumours in six Italian families segregating the same founder *SDHD* mutation Q109X

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J Med Genet 2005;42:e52 (<http://www.jmedgenet.com/cgi/content/full/42/8/e52>). doi: 10.1136/jmg.2004.030353

Background: Mutations in genes coding for the mitochondrial complex II succinate dehydrogenase (SDH) subunits cause familial neural crest derived (NCD) tumours.

Methods: Index cases from six apparently unrelated families affected by NCD tumours were analysed for mutations in the *SDHB*, *SDHC*, and *SDHD* genes.

Results: The same nonsense germline heterozygous mutation (Q109X) in exon 4 of the *SDHD* gene was found in each of the six families. Overall, 43 heterozygotes were identified. These were evaluated for the presence of NCD tumours through radiological examination of the neck, thorax, and abdomen, and measurement of urinary metanephrines and plasma chromogranin A. A novel missense *SDHD* variant, T112I, which did not segregate with the Q109X mutation and was not associated with phenotypic manifestations, was observed in one of the families. Microsatellite analysis showed a common haplotype in all individuals heterozygous for the Q109X mutation, indicating a founder effect. Overall, 18 heterozygotes were clinically affected by at least one NCD tumour. Every affected patient inherited the germline mutation from the father, confirming *SDHD* maternal genomic imprinting. Penetrance of the paternally inherited mutation progressively increased from 33% to 83% at 30 and 60 years, respectively. Affected patients showed high clinical variability, ranging from monolateral to bilateral glomus tumours variably associated or not with paragangliomas or pheochromocytomas. Loss of heterozygosity was observed in tumour cells isolated by laser capture microdissection.

Conclusions: This study shows that a single founder *SDHD* mutation is present in an area of central Italy and that this mutation is associated with widely variable interfamilial and intrafamilial expressivity.

Paragangliomas (PGLs) and pheochromocytomas (PHEOs) are neural crest derived (NCD) tumours. Several autosomal dominant inherited syndromes predispose to PGL and/or PHEO, including von Hippel-Lindau syndrome,¹ multiple endocrine neoplasia type 2,² type 1 neurofibromatosis,³ and the recently described PG/PHEO syndrome linked to mutations of genes encoding for the subunits B,⁴ C,⁵ and D⁶ of the mitochondrial complex II (succinate : ubiquinone oxidoreductase or succinate dehydrogenase; SDH).

Mitochondrial complex II consists of four subunits, each encoded by nuclear genes. Two subunits, *SDHA* and *SDHB*, constitute the hydrophilic catalytic components of the complex, while *SDHC* and *SDHD* are hydrophobic integral membrane proteins linking the catalytic subunits to the

matrix side of the mitochondrial inner membrane.⁷ Complex II is involved in the Krebs cycle, catalysing the oxidation of succinate to fumarate, and in the aerobic electron transport chain.⁸ Mutations of the *SDHA* gene cause Leigh syndrome, the clinical features of which include optic atrophy, ophthalmoplegia, nystagmus, respiratory abnormalities, ataxia, hypotonia, spasticity, and developmental delay.⁹ Germline mutations of *SDHD*, *SDHC*, and *SDHB* cause hereditary paraganglioma syndromes (HPGLs). The three subtypes of HPGLs linked to these genes are known as PGL-1, PGL-3, and PGL-4. A fourth locus has been mapped by linkage analysis but the causative gene has yet to be identified.¹⁰

PGLs can arise either from the paraganglionic bodies in the head and neck, or in the thorax or abdomen. The former are parasympathetic in origin while the latter arise from components of the sympathetic system and are usually catecholamine secreting. NCD tumours are termed PHEOs when they originate from the adrenal glands.

Recent studies seem to suggest that multiple benign head and neck PGLs may be more common in PGL-1,¹¹⁻¹³ while *SDHB* mutations (PGL-4) appear to be associated with PHEOs that may be malignant^{14 15} and possibly also with malignancies of extraparaganglial tissues.¹⁶ In studies investigating the frequency of *SDH* gene mutations in head and neck PGLs, *SDHD* is reported to be the most commonly mutated gene.¹¹ Further data are needed to define the phenotype of PGL-3, as germline mutations of *SDHC* (PGL-3) have only been described in four families with PGLs.^{5 17}

To date, at least 37 distinct *SDHD* mutations have been described.^{12 16 18-23} In the course of clinical and molecular characterisation of a series of apparently unrelated Italian patients with HPGLs, we identified a recurrent *SDHD* mutation. We present evidence that this mutation has arisen from a common ancestral chromosome. This finding provided us with the opportunity to investigate in detail the phenotypic characteristics associated with this specific molecular alteration.

METHODS

Patients and samples

Patients gave written informed consent to the study, which had been approved by the local ethics committee of our university hospital.

Index cases were part of a large series of patients affected by NCD tumours, who were thought to be affected by a germline mutation on the basis of familial history and/or the presence of multiple tumours.

Abbreviations: HPGLs, hereditary paraganglioma syndromes; LOH, loss of heterozygosity; NCD, neural crest derived; PGL, paraganglioma; PHEO, pheochromocytoma; SDH, succinate dehydrogenase

Family history was carefully reconstructed and personal clinical history was recorded for each individual. All family members participating in the study had extensive clinical evaluation and blood samples were taken for DNA analysis. Radiological examinations (mostly MRI or CT scanning, sometimes sonography) of the neck, thorax, and abdomen, and measurements of plasma chromogranin A and urinary metanephrines were performed in all participating subjects. Some patients with positive laboratory and/or radiological findings also underwent I-131-MIBG scintigraphy.

Control samples for evaluation of *SDHD* mutation frequencies were obtained after provision of informed consent from 100 unrelated healthy subjects from the same geographical area who did not have a history of NCD tumours.

Analysis of constitutional DNA

Genomic DNA was extracted from whole blood of 83 individuals from the six families under study, using the commercial kit NucleoSpin Blood L (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions.

Direct sequencing of all *SDHB*, *SDHC*, and *SDHD* exons and the exon/intron boundaries were performed in the index cases from the six families, using the primers reported in table 1. DNA sequencing analysis was limited to the mutation bearing exon in the proband's relatives.

For PCR, 200 ng of total DNA were amplified in a final volume of 25 µl. Samples were denatured for 9 minutes at 94°C, followed by 35 cycles of amplification at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 90 seconds in a Gene Amp 9700 Thermal Cycler (Applied Biosystems, Milan, Italy). Total PCR products were purified with a PCR purification kit (Qiagen, Milan, Italy) and semi-quantified in a 2% agarose ethidium bromide gel using DNA molecular weight marker XIV (Roche, Indianapolis, USA). To perform the cycle sequencing reaction, 20 ng of DNA were blended with each primer (0.8 µmol/l) in a Big Dye Terminator ready reaction mix (Applied Biosystems), denatured for 1 minute at 96°C, and then submitted to 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. A second purification with a DyeEx 2.0 Spin kit (Qiagen) was performed for Big Dye removal, then 5 µl of purified cycle sequencing product were analysed using an ABI PRISM 310 genetic analyser (Applied Biosystems).

Simple tandem repeat polymorphisms used for genotyping and haplotype analysis have been described previously.¹³ Genotypes of the patients and their parents were established

for markers *D11S5017*, *D11S5015*, *D11S5019*, *D11S5030*, and *D11S1347*. All markers were amplified using fluorescently labelled primers under standard PCR conditions. Amplicons were then submitted to ABI Prism 310 GeneScan analysis.

Laser capture microdissection

For microdissection of frozen tissue sections we used the PALM Laser Microbeam System (PALM Microlaser Technologies AG, Bernried, Germany) which enables the contact free isolation of single cells or groups of cells. Tumoral tissues submitted to this protocol were from patient III-2; family A and patient IV-3; family C. Frozen sections were mounted onto a polyethylene membrane slide and stained with haematoxylin and eosin. The microdissected cells were catapulted into the lid of a 0.5 ml reaction tube using the laser pressure catapulting technique of the instrument. For the DNA isolation, tubes were incubated overnight at 37°C with 20 µl of proteinase K digestion buffer (20 mmol/l Tris pH 8.0, 2 mmol/l EDTA, 0.5% Igepal CA-630 (Sigma) and 20 µg/ml proteinase K), then inactivated at 95°C for 10 minutes. An aliquot of this lysate was directly used for subsequent PCR and sequencing analysis to assess loss of heterozygosity (LOH).

Statistical analysis

Penetrance of *SDHD* related tumours was estimated by cumulative incidence functions, using Kaplan-Meier analysis but substituting patient age for survival time. Only index cases and relatives who had inherited the germline mutation from the father and who underwent clinical screening and radiological examinations were included for penetrance calculations.

RESULTS

Constitutional *SDHD* mutations

The same heterozygous mutation in *SDHD* exon 4 was identified in each of the six index cases (fig 1B). The mutation is a C→T substitution in a glutamine coding codon (CAA) that converts it to a stop codon (Q109X). The mutant allele is therefore predicted to encode for a truncated protein.

The mutation was subsequently identified in 37/77 relatives from the 6 families, giving a total of 43/83 carriers, including the index cases (fig 2A–F).

A novel *SDHD* heterozygous missense change (T112I) (fig 1C) was found in seven members of family B (fig 2B).

Table 1 Oligonucleotide primers for *SDHB*, *SDHC*, and *SDHD* sequencing analysis

Gene	Exon	Forward primer	Reverse primer	Amplicon length (bp)
<i>SDHB</i>	1	CGCCACCCGGGAA	GTGGCTTCTGACTTTTCCC	272
	2	GTATTCTAATTTTTTTCCTTTTGTGA	TCTTCTCAATAGCTGGCTTCA	211
	3	GCTAATACATCCAGGTGTCTCCG	CCCAACAGGAATGAAATGCTC	201
	4	GGACTGATCCGATATGGGT	AATAGCGTAACACACATAGCACTGC	256
	5	GAGTAGTCAGTGTCCAAGAAATGGG	CTTATGTTCCCTGCCAAGCC	301
	6	TCTCTCCCGTCAACAAGCTCC	AAACCAGGCCCTCAGAATG	251
	7	GAGCTTTGAGTTGAGCCAGGG	CTTCTGGCGTGTCAAGTCTG	251
	8	CTGAGGAAGGAGTTTACCCAA	TCCTGCGGCAAGTAAAGG	351
<i>SDHC</i>	1	AACCAGCAAACAGCTAGGC	AACAGTTATCAGCAAAACGTGAGG	263
	2	ATCCCTTACCCCTAAAAATAGAGA	GCTTGCAGTGAGCCGAGATC	251
	3	GCCTGGCTTGGTATTGCAA	TCTGGCTCCAGAATCCTTC	230
	4	CTATTTCAGAAATAGTTTATTTTTGCCAA	GCTGAGTTTCAAAGGAGGCG	251
	5	TAACTATGAGCAGCTGTGACAAGC	CCACTCCCTTACAGAGAAAATGT	253
	6	GGAACGTGTAATGTCTTACTTAAAT	TTCCAGGCTGGAGATAAAGAT	251
<i>SDHD</i>	1	TGACCTTGAGCCCTCAGGAACG	TCAGGGTGGGAAGACCCT	191
	2	GATCATCTAATGACTCTTTTCC	AGCAGCAGCGATGGAGAGAA	252
	3	CTTTATGAATCTGGTCTTTTTTG	CAACTATTTTGAATTGCTATAC	338
	4	AGTGGAGTGGCAAATGGAGA	AATGGCATGACAAAGCAGAGG	360

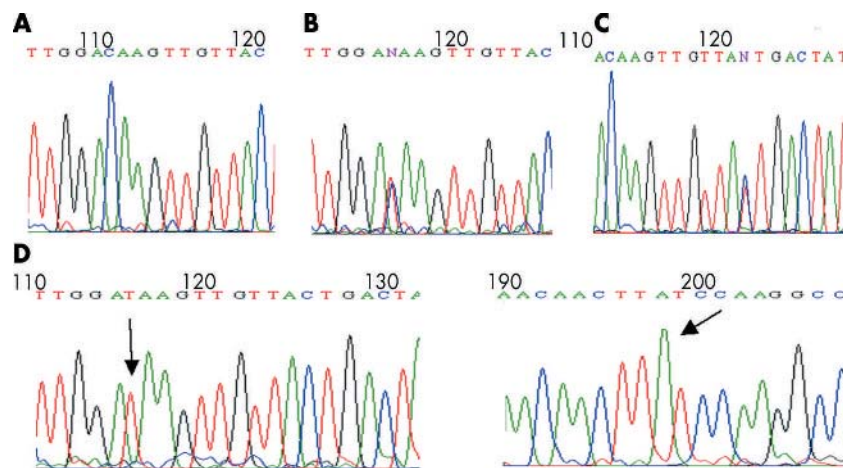


Figure 1 Electropherograms showing the exon 4 sequence of the *SDHD* gene: (A) wild type sequence; (B) Q109X mutation; and (C) T112I substitution. In panel (D), forward and reverse sequences of tumour DNA showing LOH are shown. The arrows indicate the positions of the modified nucleotides.

This change was not present in any of 100 control subjects originating from the same area.

Haplotype analysis

Although no apparent relationship among the six families segregating the Q109X mutation could be found upon careful pedigree reconstruction, all families originated from a small area in the region of Tuscany. Therefore, we performed haplotype analysis to verify if the occurrence of the mutation could be due to a founder effect. Analysis of *SDHD* linked markers showed that a common haplotype, spanning a region of 304 kb that includes the *SDHD* gene, was present in all heterozygotes (fig 2). No recombination events were observed. In family B the T112I missense change segregated independently of the Q109X mutation (fig 2B).

Loss of heterozygosity

Sequencing analysis performed on DNA obtained from tumoral tissues was not indicative of LOH. Microdissected tumoral tissues from patient III-2, family A and patient IV-3; family C showed loss of wild type allele in both the samples (fig 1D).

Clinical evaluation

The clinical characteristics of Q109X mutation carriers are shown in table 2. Laboratory and radiological screening revealed the presence of one or multiple NCD tumours in 18 patients (8 male and 10 female patient). Each affected patient had inherited the mutation bearing chromosome from the father, while none of the carriers who inherited the mutant allele from the mother was affected by a NCD tumour.

Overall, we were able to document 48 NCD tumours. The most frequent tumour localisation was the neck, followed by the abdomen. Plasma chromogranin A levels were elevated in 10 of 14 patients harbouring a NCD tumour. Urinary metanephrines were elevated in six patients, five of whom were affected by an abdominal NCD tumour and one by a thoracic paraganglioma. Patients apparently affected only by a neck paraganglioma did not show any increase in urinary metanephrines. Multiple or recurrent NCD tumours were found in 15 of 18 patients. The mean age of clinical presentation was 39 years (range 14–69).

Radiological or laboratory evidence for the presence of NCD tumours was not found in any of the T112I heterozygotes identified in family B.

Of the 23 patients who inherited the germline mutation from the father, three were unaffected (family B, III-6; family C, IV-20 and family F, V-6), and two (family C, II-1 and

III-19), aged 76 and 52 years respectively, refused clinical evaluation. The other 18 patients had clinical and biochemical diagnosis of NCD tumours. Age related penetrance based on symptomatic and asymptomatic tumours is shown in fig 3. Penetrance values progressively increased from 33% to 83% at 30 and 60 years respectively, and 50% of heterozygotes were affected by 33 years of age.

DISCUSSION

SDHD mutations have been shown to predispose to PGL-1. In our study, we evaluated six families affected by PGL-1 caused by the same founder mutation, Q109X. This is the first study demonstrating the existence of a PGL-1 syndrome due to a founder effect in Italy. *SDHD* founder mutations have been found in the Netherlands (D92Y, L139P, and L95P)^{24, 25} and in several families in the USA (P81L),⁶ but not in other studies investigating families from Australia, the UK, Germany/Poland, and Spain.¹²

The Q109X mutation has previously been described in only one family, the pedigree of which was characterised by the presence of two heterozygous individuals who developed carotid body tumours.¹³ We were able to collect clinical and genetic information from a large number of individuals belonging to the six Italian families with the Q109X mutation, which allowed us to perform genotype phenotype correlations. Haplotype analysis demonstrated that the mutation was derived from a common ancestral chromosome. Pedigree analysis showed that the PGL-1 syndrome is present only when the mutation is paternally inherited, thus confirming the maternal imprinting of the *SDHD* gene.²⁶ Family E presented only one affected patient and did not contribute to the study of intrafamilial variability. Nevertheless, it constitutes a good example of maternal imprinting, as none of the affected woman's offspring, aged 44, 41, 33, and 31 years, have been affected.

The most striking finding of our study is the wide variability in the clinical picture of the heterozygous individuals who had received the mutant gene from the father. Variability was observed in the age of onset, the type of clinical presentation, and number, localisation, and secreting properties of the NCD tumours. There was also intrafamilial variability in the phenotype of similar tumours. For instance, in family A, the index patient developed multiple catecholamine secreting chromaffin tumours, including bilateral PHEOs, while his sister was affected by a large (10 cm in diameter) monolateral, non-secreting PHEO at the age of 31 years.

Wide intrafamilial difference in age at presentation of glomus tumours was present, which could be partially

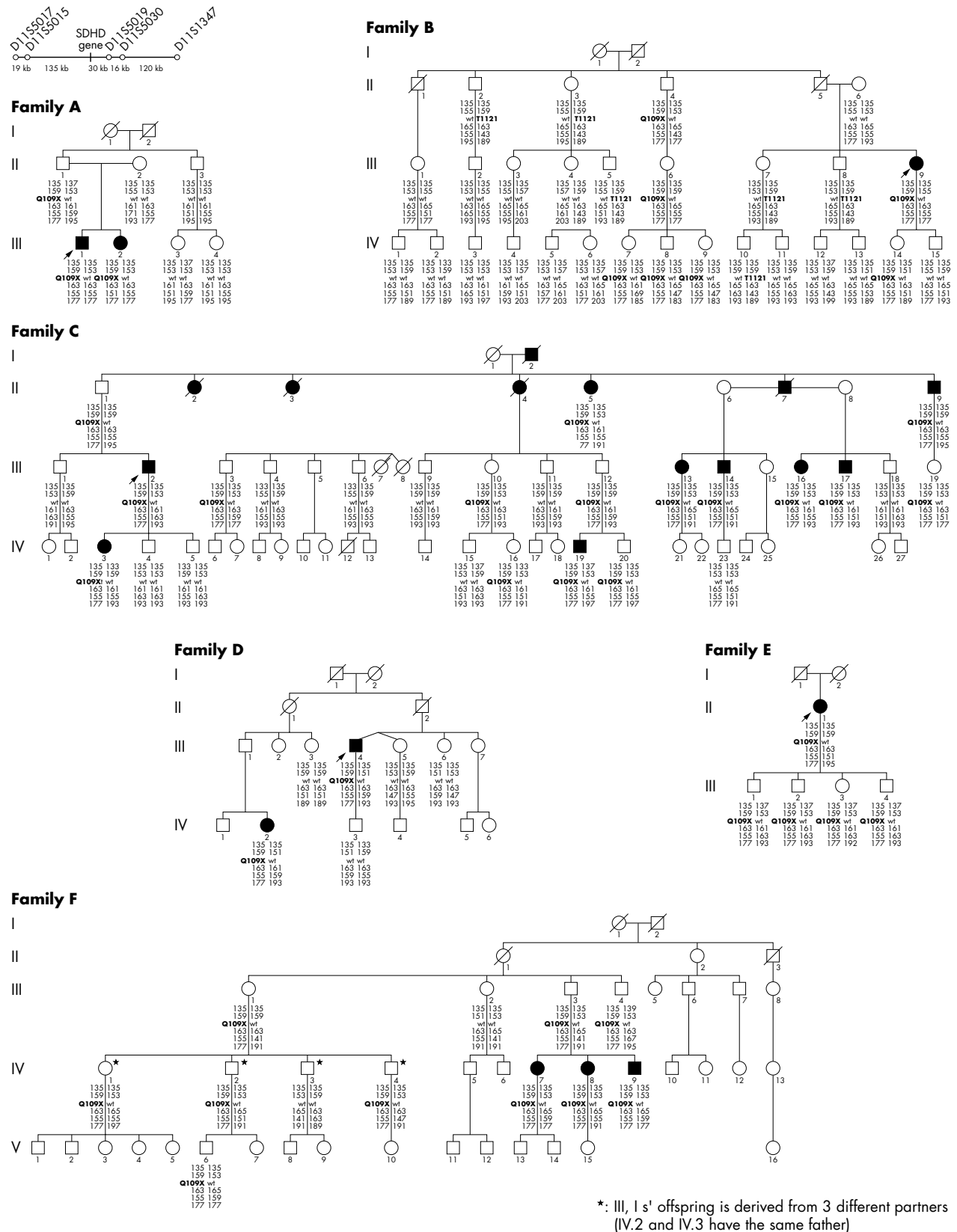


Figure 2 Pedigrees of the families segregating the Q109X mutation. Haplotypes are shown below each symbol; from top to bottom, alleles at the following loci are shown: *D11S5017*, *D11S5015*, *SDHD* (codon 109 or 112) *D11S5019*, *D11S5030*, and *D11S1347*. The offspring of III-1 are those of three different partners (IV-2 and IV-3 have the same father).

Table 2 Phenotypic manifestations in Italian PGL-1 families segregating the Q109X *SDHD* mutation

Family	Subject	Age at onset, years	Phenotype	
A	III-1	20	Abdominal paragangliomas + bilateral PHEOs	
	III-2	31	Right jugular paraganglioma + left PHEO	
B	III-9	43	Bilateral carotid body paragangliomas + bladder paraganglioma	
C	I-2	NK	Died at 51 years, hemiplegic, hypertensive crises (not personally evaluated)	
	II-1	NK	Not clinically evaluated	
	II-2	NK	Referred neck masses (not personally evaluated)	
	II-3	NK	Referred neck masses (not personally evaluated)	
	II-4	NK	Referred neck masses (not personally evaluated)	
	II-5	69	Bilateral carotid body paragangliomas	
	II-7	NK	Died at 54 years during a hypertensive crises (not personally evaluated)	
	II-9	66	Bilateral carotid body paragangliomas	
	III-2	48	Bilateral carotid body paragangliomas + mediastinal paraganglioma	
	III-13	14	Bilateral carotid body paragangliomas + jugular paraganglioma	
	III-14	52	Right glomus tumour + cerebral cavernous angiomas	
	III-16	31	Right PHEO	
	III-17	41	Left glomus tumours + abdominal paraganglioma	
	III-19	NK	Not clinically evaluated	
	IV-3	25	Bilateral carotid body paragangliomas	
	IV-19	33	Left glomus tumour	
	D	III-4	57	Left jugular-timpanic + bilateral glomus tumours
		IV-2	23	Bilateral carotid body paragangliomas
	E	II-1	65	Bilateral carotid body paragangliomas + paratracheal paraganglioma
F	IV-7	33	Bilateral carotid body paragangliomas + bilateral vagal paragangliomas	
	IV-8	25	Laryngeal paraganglioma + right glomus tumour + right vagal-giugular paraganglioma + left vagal paraganglioma	
	IV-9	21	Bilateral carotid body paragangliomas	

NK, not known.

accounted for by tumour dimension. In family C, age at onset ranged from 25 years in patient IV-3 to 69 years in patient II-5, who was asymptomatic and diagnosed only in the course of the family screening. In this family, at least two other patients showed particular characteristics. Patient III-16 was affected by a right adrenal PHEO at the age of 31 years for which she underwent surgery; 14 years after the surgery, this seems to be the only tumour so far developed. This patient's halfbrother (III-14), in addition to having a monolateral glomus tumour, was affected by multiple cavernous cerebral angiomas (CCA). Such lesions have not been previously described in PGL-1 and might represent a coincidental finding related to mutations associated with CCA.

A common feature of the NCD tumours in the families under study is the absence of malignancy, thus confirming the lack of biological aggressiveness of PGL-1 tumours.^{12, 13}

Of 14 patients presenting a NCD tumour, four had normal chromogranin A plasma levels. Increased levels of urinary metanephrines were found only in patients with one or more documented abdominal or thoracic PGLs. Nevertheless, patient III-2 in family A and patient III-2 in family C had normal urinary metanephrines in spite of the presence of a left PHEO and a thoracic PGL, respectively. Therefore, plasma

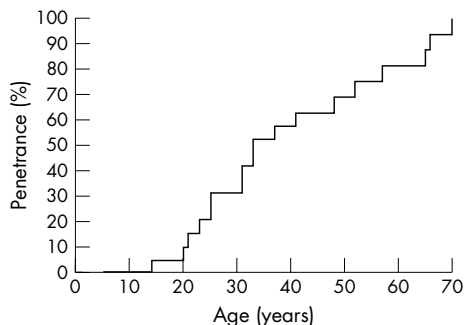
chromogranin A and urinary metanephrine assays were shown to be useful laboratory indexes for clinical assessment of these patients, although their sensitivity does not reach 100%.

Of 21 patients who inherited the Q109X germline mutation from the father, 18 had a diagnosis of NCD tumours upon clinical evaluation. Three subjects were found to be unaffected by NCD tumours upon through screening; a 45 year old woman, an 18 year old man, and a 5 year old boy. Penetrance values are high, with 50% penetrance by 33 years of age, which is in agreement with data obtained through the analysis of 35 European subjects harbouring different *SDHD* mutations.¹⁶

Our data provide evidence of LOH in tumoral tissues, and agree with previous reports.²⁷ Loss of maternal wild type allele was detectable only in microdissected tumoral samples that enable isolation of neoplastic chief cells exclusively, thus confirming the cellular heterogeneity of these tumors.

At present the role, if any, of the maternal allele in preventing tumour development is unknown, but LOH in the tumour might be one factor explaining variability of penetrance and expressivity in PGL-1. Nevertheless, it should be noted that the *SDHD*-containing region on 11q23 is extremely gene rich,²⁸ and therefore LOH might target a nearby gene. The extent of LOH might be of importance. Indeed, a recent study suggests that an exclusive loss of the entire maternal chromosome 11 is the most common second hit in *SDHD* linked PGLs.²⁹ This could cause the selective loss of one or more imprinted genes in the 11p15 region. Further investigation of microsatellite loci and single nucleotide polymorphisms will be useful to better define the extent of the allelic loss on chromosome 11.

Different mutations affecting other loci may also be involved in tumorigenesis in the different targets of the PGL-1 syndrome. Phenotypic expression of the Q109X and other *SDHD* mutations could also be modulated by variants at *SDHD* unlinked loci acting as genetic modifiers. It has been suggested that environmental factors, such as oxygen tension

**Figure 3** Age related penetrance for *SDHD* mutation carriers (n = 21).

and therefore altitude, could influence penetrance and expressivity of *SDH*-linked syndromes.³⁰ Our six families, at least for the generations we examined, have always lived in a relatively restricted area of Tuscany, in places where altitude ranges from 29 to 61 metres above sea level. Therefore, we could not document any correlation between disease expressivity and atmospheric pressure in our series. In addition, we did not observe correlations with smoking behaviour (data not shown).

In family B, seven subjects had a novel aminoacid substitution in *SDHD*, T112I, caused by a C→T transition. While this was not detected in a series of 100 control subjects living in the same area, it was found to be unassociated with phenotypic manifestations of the PGL-1 syndrome in any of the carriers. Threonine at codon 112 is conserved in *Mus musculus*, *Rattus norvegicus*, and *Xenopus laevis*, but not in *Drosophila melanogaster*, *Caenorhabditis elegans*, or *Schizosaccharomyces pombe*. Therefore, T112I is probably a rare polymorphism, although a functional effect in *SDHD* activity associated with low penetrance is also possible.

In conclusion, in this study we demonstrate that the PGL-1 syndrome in families originating from an area of central Italy is predominantly caused by a single founder *SDHD* mutation. Disease transmission was confirmed to be under parent of origin effect. While penetrance of the mutation is high, its phenotypic expressivity shows wide interfamilial and intra-familial variability. The causes of such variability and the molecular and genetic mechanisms leading to tumour formation in individuals heterozygous for such mutations will have to be further investigated.

ACKNOWLEDGEMENTS

We thank all the patients who participated in this study. We wish to thank Professors M Pazzagli and C Orlando, Department of Clinical Physiopathology, Clinical Biochemistry Unit, for advice and suggestions on laboratory technologies. We are grateful to Dr M Rotondi, Department of Clinical Physiopathology, Section of Endocrinology, for his assistance for statistical analysis. This study was conducted on behalf of ENS@T (European Network for the Study of Adrenal Tumours). This work was partly supported by grants from MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca) (prot.2004069534) and Department of Clinical Pathophysiology, University of Florence. M Genuardi gratefully acknowledges the support of Ente Cassa di Risparmio di Firenze to the Fiorgen Foundation.

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Competing interest: there are no competing interests.

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Received 23 December 2004

Revised 9 March 2005

Accepted 11 March 2005

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