Paragangliomas: Clinical Picture

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Chapter 4

Succinate dehydrogenase (SDH)-deficient pancreatic neuroendocrine tumor expands the SDH-related tumor spectrum

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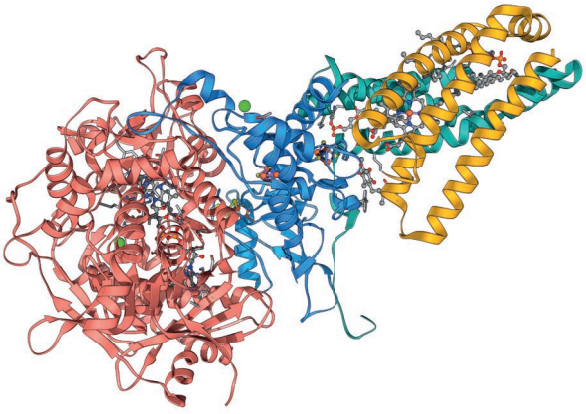

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Abstract

Context: Mutations in genes encoding the subunits of succinate dehydrogenase (SDH) can lead to pheochromocytoma/paraganglioma formation. However, *SDH* mutations have also been linked to nonparaganglionic tumors.

Objective: The objective was to investigate which nonparaganglionic tumors belong to the SDH-associated tumor spectrum.

Design: This was a retrospective cohort study.

Setting: The setting was a tertiary referral center.

Patients: Patients included all consecutive *SDHA/SDHB/SDHC* and *SDHD* mutation carriers followed at the Department of Endocrinology of the Leiden University Medical Center who were affected by non-pheochromocytoma/paraganglioma solid tumors.

Main Outcome Measures: Main outcome measures were SDHA/SDHB immunohistochemistry, mutation analysis, and loss of heterozygosity analysis of the involved *SDH*-encoding genes.

Results: Twenty-five of 35 tumors (from 26 patients) showed positive staining on SDHB and SDHA immunohistochemistry. Eight tumors showed negative staining for SDHB and positive staining for SDHA: a pancreatic neuroendocrine tumor, a macroprolactinoma, two gastric gastrointestinal stromal tumors, an abdominal ganglioneuroma and three renal cell carcinomas. With the exception of the abdominal ganglioneuroma, loss of heterozygosity was detected in all tumors. A prolactinoma in a patient with a germline *SDHA* mutation was the only tumor immunonegative for both SDHA and SDHB. Sanger sequencing of this tumor revealed a somatic mutation (p.D38V) as a likely second hit leading to biallelic inactivation of SDHA. One tumor (breast cancer) showed heterogeneous SDHB staining, positive SDHA staining and retention of heterozygosity.

Conclusions: This study strengthens the etiological association of *SDH* genes with pituitary neoplasia, renal tumorigenesis and gastric gastrointestinal stromal tumors. Furthermore, our results indicate that pancreatic neuroendocrine tumor also falls within the SDH-related tumor spectrum.

Introduction

Mutations in any one of the succinate dehydrogenase (SDH) complex subunits (*SDHA*, *SDHB, SDHC*, *SDHD* and *SDHAF2*) can lead to formation of pheochromocytoma (PCC)/paraganglioma (PGL). Heterozygous germline mutations of *SDHB*, *SDHC* and *SDHD* cause the well-characterized familial PCC-PGL syndromes known as PGL4, PGL3 and PGL1, respectively.[1,2] The gene for PGL2 syndrome has been identified as *SDHAF2* (SDH5).[3] The *SDHA*, *SDHB*, *SDHC* and *SDHD* genes encode for the four subunits of SDH (mitochondrial complex II), a key respiratory enzyme which links the Krebs cycle and the electron transport chain.[4] The *SDHAF2* gene encodes SDH complex assembly factor 2 (SDHAF2), essential for flavination of the SDHA protein and SDH enzyme activity.[2] If mutations occur in the *SDHA, SDHB, SDHC*, *SDHD* or *SDHAF2* genes with corresponding loss of the wild-type allele or a second inactivating mutation, SDHB immunohistochemical staining will become negative.[5] This negative staining for SDHB is now a validated and highly sensitive marker for germline mutations of any of the *SDH* subunits and is a broadly accepted indication of pathogenicity of an *SDH* mutation.[6,7] In addition, SDHA immunohistochemistry is a proven marker for *SDHA* mutations, showing loss of immunoreactivity exclusively in *SDHA*-mutated tumors, while non-*SDHA*-mutated tumors, including *SDHB*, *SDHC*, *SDHD* and *SDHAF2*-mutated cases, show positive SDHA staining.[5,8]

SDHA, SDHB, SDHC and *SDHD* mutations have also been linked to gastrointestinal stromal tumor [4,9] and renal-cell carcinoma.[10-15] SDH-deficient renal carcinoma has been accepted as a provisional entity in the 2013 International Society of Urological Pathology Vancouver Classification. Gill *et al.* studied 36 SDH-deficient renal carcinomas and showed that these carcinomas had a strong relationship with *SDH* germline mutation.[16] In addition, pituitary adenomas have been reported to be associated with *SDHA*, *SDHB*, *SDHC* and *SDHD* mutations.[12,17-20] However, other nonparaganglionic tumors may belong to the SDH tumor spectrum. To address this issue, we investigated all nonparaganglionic tumors affecting patients included in the Leiden SDH Mutation Carrier Registry.

Subjects and methods

Subjects

All consecutive *SDHA*, *SDHB*, *SDHC* and *SDHD* mutation carriers followed at the Department of Endocrinology of the Leiden University Medical Center who were affected by non-PCC/PGL solid tumors and who gave written informed consent were included. Of the three *SDHA* mutation carriers, one had a non-PCC/PGL tumor. Of the 54 *SDHB* mutation carriers, seven had non-PCC/PGL tumors, of which six were available for investigation. Of the 239 *SDHD* mutation carriers, 22 were affected by non-PCC/PGL tumors. Histological material was

unavailable from one patient and as two additional patients underwent only radiological follow-up, no biopsy or surgically-resected material was available. Of the four *SDHC* mutation carriers, one was affected by a non-PCC/PGL tumor. However, this patient did not provide written informed consent and was therefore excluded. In total, 26 patients with 35 non-PCC/PGL tumors were included.

Tissue samples

Archival specimens of tumor and normal formalin-fixed paraffin-embedded (FFPE) tissues were provided by the hospitals where the patients underwent surgery. Clinical and genetic characteristics of the patients are detailed in Supplemental Table 1.

SDHA/SDHB immunohistochemistry

All nonparaganglionic tumors were analyzed with SDHA and SDHB immunohistochemistry (IHC). FFPE tissue sections of 4 µm thickness were stained with commercially available antibodies: mouse monoclonal Ab14715 antibody (Mitosciences, Abcam, Cambridge, UK; 1:500 dilution) against SDHA and rabbit polyclonal HPA002868 antibody (Sigma–Aldrich Corp, St. Louis, MO, USA.; 1:400 dilution) against SDHB. Stainings were performed on an automatic Ventana Benchmark Ultra System (Ventana Medical Systems Inc. Tuscon, AZ, USA) using the Ultraview DAB detection system, following heat-induced epitope retrieval with Ventana Cell Conditioning 1 (pH 8.4) at 97°C for 52 and 92 minutes, respectively.

Loss of heterozygosity (LOH) analysis

DNA isolation from SDHB and/or SDHA immunonegative tumors was carried out using standard procedures after manual microdissection. All tumor samples were estimated to contain at least 80% neoplastic cells. LOH analysis of SDHB immunonegative/SDHA immunopositive tumors was performed using polymorphic microsatellite markers flanking either the *SDHB* (one surrounding a microsatellite located at UCSC chr1:17,417,100 and D1S507) or the *SDHD* (D11S5015, D11S5017, D11S5019 and D11S1347) gene. Tumor DNA and fluorescently-labeled primers (Invitrogen; primer sequences available on request) underwent 35 cycles of PCR at an annealing temperature of 60°C. Amplified products were analyzed, along with LIZ 500 size standard (Applied Biosystems, Bleiswijk, the Netherlands), using capillary electrophoresis on an ABI 3130-XL genetic analyzer (Applied Biosystems). Data were analyzed using GeneMarker Software (Soft-Genetics LLC, State College, PA, USA).

Mutation screening

From SDHB immunonegative/ SDHA immunopositive tumors without LOH or lack of informative (centromeric or telomeric) markers, the full coding sequence, including

intron–exon boundaries, was screened for *SDHD* and *SDHB* mutations at the somatic level either by Sanger (direct) sequencing in forward and reverse orientation or by using an Ion AmpliSeq Custom Panel sequenced on the Ion Torrent Personal Genome Machine (PGM; Life Technologies) respectively, as previously described.[12,21] In addition, Sanger sequencing was also used to confirm the presence of the known mutations in the tumors, and to investigate the occurrence of loss of the wild-type allele in all cases with immunonegative SDHB staining.

Results

An overview of the immunohistochemical and sequencing results is shown in Supplemental Figure 1. Thirty-five nonparaganglionic tumors from 26 *SDH* mutation carriers were analyzed in the current study (Supplemental Table 1). No further analysis was carried out in 25 tumors displaying SDHB and SDHA immunopositivity, with the exception of one growth hormone producing pituitary adenoma (case 8), because this analysis was conducted previously as reported in Papathomas *et al.*[12] The 25 SDHB/SDHA immunopositive tumors obtained from 15 *SDHD*- and four *SDHB* mutation carriers, encompassed papillary thyroid carcinoma, melanoma, bladder cancer, endometrial cancer, prostate cancer, testicular cancer, meningioma, basal cell carcinoma, and sebaceous gland carcinoma of the eyelid (Supplemental Table 1). The clinicopathological and molecular genetic characteristics of the remaining 10 tumors displaying SDHB immunonegativity (n=9) or heterogeneous immunoexpression pattern (n=1) are displayed in Table 1. These tumors occurred in seven patients, of which four harbored an *SDHD* germline mutation, two harbored an *SDHB* germline mutation, and one harbored an *SDHA* germline mutation.

In particular, nine tumors showed loss of SDHB expression. Eight of these displayed positive staining for SDHA: a pancreatic neuroendocrine tumor (NET) (case 1; Figure 1A, B, C), a macroprolactinoma (case 2), an abdominal ganglioneuroma (case 5), two gastric gastrointestinal stromal tumors (GIST) (cases 4 and 6) and three renal cell carcinomas (case 6). One tumor (case 7) showed loss of SDHB and SDHA expression. Seven of the nine SDHB immunonegative tumors showed LOH for at least one of the microsatellite markers, indicating biallelic inactivation of the given *SDH* gene (Table 1). Loss of the wild-type allele was also confirmed by the Sanger sequencing results (Figure 1D, E). Sanger sequencing of the single SDHB/SDHA-immunonegative macroprolactinoma (case 7) revealed a somatic SDHA mutation (p.D38V), along with the germline *SDHA* mutation (p.R31X). In conclusion, eight tumors fulfilled the criteria of biallelic inactivation of the given *SDH* gene (Table 1). In contrast, the SDHB immunonegative abdominal ganglioneuroma (case 5) showed retention of heterozygosity, similarly to the single tumor (breast cancer, case 3) exhibiting a heterogeneous SDHB immunoexpression pattern.

Table 1. Clinicopathological and molecular genetic characteristics of nonparaganglionic tumors displaying SDHB immunonegativity or heterogenous immunoexpression pattern

Case	Age[a]/sex	Germline *SDH* mutation ¶	Tumors observed (age at detection, y)	SDHB IHC	SDHA IHC	Second inactivation hit ¶ ¶	Status at last follow-up (age, y)
1	56/M	*SDHD* p.Asp92Tyr c.274G>T	pNET (56) Oligodendroglioma (57) GCT (L+R) (56) GVT (R) (56)	Neg Pos	Pos Pos	LOH#	Died (64)
2	61/M[b]	*SDHD* p.Asp92Tyr c.274G>T	Macroprolactinoma (61) PCC (R) (61) GJTT (R) (60) GCT (L) (60) GVT (L+R) (60)	Neg	Pos	LOH	AWED (69)
3	38/F[c]	*SDHD* p.Pro81Leu c.242C>T	Breast cancer (38) GCT (L) (38)	Hetero-genous^	Pos	ROH	Died due to breast cancer (41)
4	55/F	*SDHD* p.Asp92Tyr c.274G>T	Gastric GIST (55) GCT (L+R) (50) GVT (L) (50)	Neg	Pos	LOH#	AWED (64)
5	42/M	*SDHB* c.423+1G>A	Abdominal ganglioneuroma (42) GVT (R) (42)	Neg	Pos	ROH	AWED (50)
6	45/M[d]	*SDHB* c.423+1G>A	RCC L foci 1 (45) RCC L foci 2 (45) RCC R (45) Gastric GIST (45) No PGL	Neg Neg Neg Neg	Pos Pos Pos Pos	LOH LOH LOH LOH	AWED (47)
7	49/F	*SDHA* p.Arg31X c.91C>T	Macroprolactinoma (49) GCT (R) (26) GCT (L) (49) Meningiomas (49)	Neg Pos	Neg Pos	p.D38V	AWD (meningioma) (65)

Abbreviations: *M* male; *F* female; *IHC* immunohistochemistry; *pNET* pancreatic neuroendocrine tumor; *GCT* glomus caroticum tumor; *GVT* glomus vagale tumor; *GJTT* glomus jugulotympanicum tumor; *PGL* paraganglioma; *PCC* pheochromocytoma; *RCC* renal cell carcinoma; *GIST* gastrointestinal stromal tumor; *L* left; *R* right; *AWD* alive with (non-paraganglionic) disease; *AWED* alive without evidence of disease other than head and neck PGL; *Pos* positive; *Neg* negative; *ROH* retention of heterozygosity; *LOH* loss of heterozygosity; *LOH#* only one marker (centromeric or telomeric) was informative in each tumor as indicative of LOH. Sanger (direct) sequencing showed loss of the wild-type allele.

a Age at diagnosis of non-PCC/PGL tumor.
b Patient previously described by Papathomas *et al.*[12]
c Patient also carrier of a germline breast cancer 1 (*BRCA1)* mutation.
d Patient previously described by Gill A *et al.*[16]

¶ The germline mutation was documented in all tumors.
¶¶ Loss of wild-type allele or somatic mutation.
^ Heterogenous is defined as granular cytoplasmic staining combined with a cytoplasmic blush lacking definite granularity or completely absent staining in the presence of an internal positive control throughout the same slide.

Case 1 originally presented with a pancreatic mass that was eventually diagnosed as a pancreatic NET. An octreotide scan performed to further evaluate the pancreatic mass led to the subsequent detection of head and neck PGLs. Head/neck magnetic resonance imaging (MRI) confirmed the presence of the latter. Genetic analysis identified a germline *SDHD* mutation. The patient's twin sister and brother are both affected by PGLs, as are the father and an uncle (Figure 2). After confirmation of the germline *SDHD* mutation, follow-up with urinary analysis for catecholamine excess and head and neck MRIs was initiated. One year later, a brain MRI was performed due to visual field complaints; it showed a (histologically proven) low-grade oligodendroglioma in the right frontal lobe. This resulted in the person being affected by epilepsy seizures. The patient died at the age of 64 years, due to the complications of a pneumosepsis with pleural empyema and left hydropneumothorax. The LOH analysis together with SDHB immunonegativity, strongly suggests that this tumor is most likely caused by the germline *SDHD* mutation.

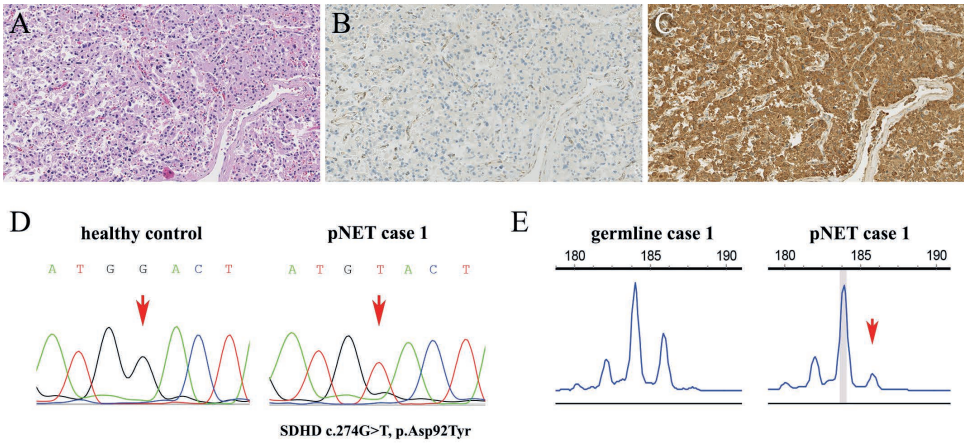

Figure 1. (A) Hematoxylin and eosin staining of the pancreatic neuroendocrine tumor arising in a patient carrying a germline *SDHD* c.274G>T (p.Asp92Tyr) mutation; **(B)** SDHB immunohistochemistry (IHC) displaying loss of expression in the neoplastic cells with normal (endothelial) cells serving as positive internal controls; **(C)** SDHA IHC showing immunopositivity in both neoplastic and non-neoplastic cellular compartments; **(D)** Sequencing chromatograms of healthy germline tissue and tumor DNA. Mutational analysis revealed the germline *SDHD* c.274G>T (p.Asp92Tyr) mutation in the pancreatic neuroendocrine tumor. Note the absence of the wild-type allele indicating loss of heterozygosity (LOH); and **(E)** Loss of heterozygosity (LOH) electropherogram. Heterozygosity was lost only for a microsatellite marker (D11S5019) telomeric to the SDHD locus. The red arrows indicate the allele with relative loss. Heterozygosity was retained for a microsatellite marker (D11S5017) centromeric to the SDHD locus, while the patient was homozygous (not informative) for another marker (D11S5015) on the centromeric side (LOH electropherograms not shown).

Abbreviation: *pNET* pancreatic neuroendocrine tumor.

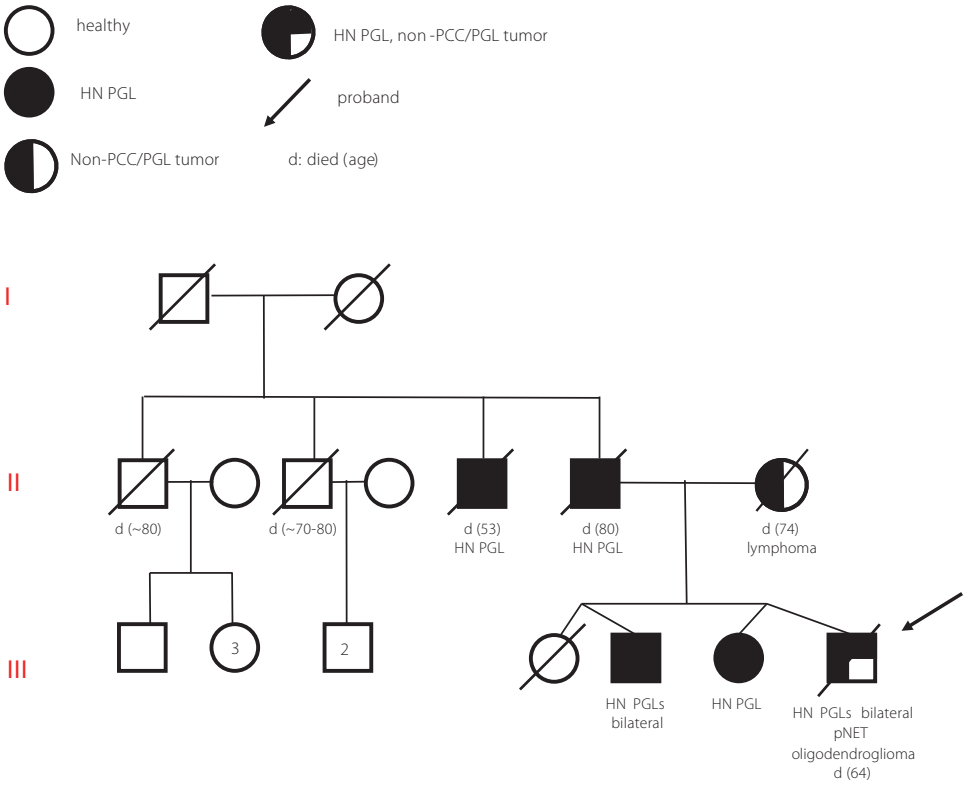

Figure 2. Pedigree from case 1, a patient with a germline *SDHD* (p.Asp92Tyr) mutation with bilateral paragangliomas, a pancreatic neuroendocrine tumor and an oligodendroglioma.

Abbreviations: *HN PGL* head and neck paraganglioma; *non-PCC/PGL* non-pheochromocytoma/paraganglioma; *pNET* pancreatic neuroendocrine tumor.

Discussion

Our initial immunohistochemical analysis of 35 nonparaganglionic tumors from 26 *SDH* mutation carriers identified and excluded 25 SDHB and SDHA immunopositive tumors. Immunohistochemical and molecular genetic data from eight SDHB immunonegative tumors confirm that a wide range of nonparaganglionic tumors fall within the SDH-related tumor spectrum and suggest the pancreatic NET may also expand this spectrum. In particular, we present strong supporting evidence indicating that the pancreatic NET described here very likely arose due to a germline *SDHD* mutation and is therefore a constituent of the *SDHD*-associated tumor spectrum. This is the first report of an association between a germline *SDHD* mutation and pancreatic NET and as such it expands the spectrum of hereditary pancreatic NETs, previously only attributable to multiple endocrine

neoplasia type 1 (MEN1), Von Hippel-Lindau disease (VHL), neurofibromatosis 1 (NF-1) and the tuberous sclerosis complex (TSC).[22]
The occurrence of a pancreatic NET in a *SDHD* mutation carrier is rare. Of the 239 patients with a germline *SDHD* mutation enrolled in the Leiden SDH Mutation Carrier Registry, only one of 22 patients suffering from a nonparaganglionic tumor was affected by a pancreatic NET. Despite the rarity, this finding might have potential implications for the surveillance of patients with a germline *SDHD* mutation. In The Netherlands, the surveillance protocol for *SDHD* mutation carriers includes urinary analysis for catecholamine excess every two years and MRI of the head/neck region every three years. Abdominal imaging is only advised when there is evidence of catecholamine excess. The addition of pancreatic NET to the SDH-related tumor spectrum suggests that it might be advisable to amend surveillance protocols, with the addition of standard abdominal imaging studies. Because the latter are not currently included in surveillance protocols in The Netherlands, the possibility that other patients in our registry carry undetected pancreatic NETs cannot be ruled out. However, given the rare occurrence rate in our study, further studies are needed to definitely amend surveillance protocols.

This study also included a patient with a germline *SDHA* mutation and an associated pituitary adenoma. This case, along with an additional case previously described by Papathomas *et al.*[12], suggests an important role for SDH mutations in hypophyseal tumorigenesis. These cases, together with the large patient cohort described by Dénes *et al.*[20], not only support a causative role of SDH genes in pituitary adenoma formation, but also highlight genotype-phenotype correlations in this fast-moving endocrine field. To date, 25 pituitary adenoma cases have been described occurring in association with confirmed germline *SDH* mutations/variants (Table 2).[12,17-20,23-26] Most of these tumors are prolactinomas, nonfunctioning adenomas or growth-hormone secreting macro-adenomas, with variable ages at diagnosis ranging from 15 to 84 years. It is now clear that germline *SDH* mutations are also a component of the familial spectrum of pituitary adenomas comprising *F*amilial *I*solated *P*ituitary *A*denoma (FIPA) (germline inactivating aryl hydrocarbon receptor interacting protein (*AIP*) mutations), Carney complex (germline inactivating *PRKARIA* mutations), *M*ultiple *E*ndocrine *N*eoplasia, type 1 (germline inactivating *MEN1* mutations) and *M*ultiple *E*ndocrine *N*eoplasia, type 4 (germline inactivating *CDKN1B* (p27/KIP1) mutations).[27-29]
Biallelic *SDHA* inactivation has been documented in both paragangliomic tumors and GISTs arising in patients harboring a germline *SDHA* mutation.[30] To the best of our knowledge, this is the first pituitary adenoma with proven biallelic inactivation in a patient with a germline *SDHA* mutation. Dwight *et al.* described a family in which a germline *SDHA* mutation was associated with a PGL in the proband, as well as a pituitary nonfunctioning macroadenoma in the proband's son. SDHA immunohistochemistry confirmed loss of expression in both

tumors. However, biallelic SDH inactivation was not detected in the pituitary adenoma; only paradoxical loss of the mutated allele was detected.[19] Dénes *et al.* demonstrated LOH in the pituitary adenomas of 3 *SDHB* patients, but there was ROH in the pituitary adenomas of two *SDHA*-mutated patients.[20] Gill *et al.* detected two inactivating SDHA mutations in a 62-year-old man with a prolactin-producing tumor, but neither of these mutations was present in the germline.[31] In an effort to identify the underlying pathogenic mechanism by which *SDH* mutations lead to pituitary tumor development, Xekouki *et al.* studied the pituitary in *Sdhb*$^{+/-}$ mice and provided evidence that pituitary hyperplasia in SDH-deficient cells may be the initial abnormality in the cascade of events leading to true adenoma formation.[26] These data unravel critical aspects related to hypophyseal pathobiology and further add to the understanding of the tumorigenic process.

In contrast, other tumor types, eg, bladder cancer, melanoma, prostate cancer and papillary thyroid cancer, retained SDHB/SDHA protein expression, suggesting that these tumors are not part of the SDH-associated tumor spectrum. To extend, the biological nature of heterogeneous breast cancer (case 3) in this particular genetic context (ie, *SDHD* and *BRCA-1*) remains elusive. Along these lines, the SDHB immunonegative abdominal ganglioneuroma (case 5) displayed ROH in the absence of additional mutations, strongly suggesting an alternative mechanism of SDHB protein loss other than loss of genomic regions encompassing the *SDHB* locus and/or a second 'exonic' somatic event. An alternative mechanism could be *SDHC* promoter hypermethylation.[32,33] Nevertheless, a limitation of the current study concerns the lack of methylation analysis for the promoter of *SDHC* gene.

In conclusion, the current study expands the SDH-related tumor spectrum and identifies pancreatic NET as a new component of this spectrum. This study also strengthens the etiological association of *SDH* genes with pituitary neoplasia, renal tumorigenesis and gastric GISTs as revealed in the Leiden SDH Mutation Carrier Registry. These findings may have implications for the surveillance protocol for patients with a germline *SDHD* mutation. In this context, further studies are warranted to elucidate the role of the disruption of the Krebs cycle in familial and sporadic pancreatic neuroendocrine tumorigenesis.

Table 2. Germline *SDH* mutations/variants and pituitary adenomas reported in the literature

Case	Age^/sex	Functional classification of PA	Germline *SDH* mutation	Biallelic SDH inactivation in PA	PGL/PCC	Reference
1	30/M	Pituitary nonfunctioning macroadenoma	*SDHA* c.1873C>T p.His625Tyr	Paradoxical loss (LOH) of the mutated allele	None	19
2	27/M	Pituitary prolactinoma, size NA	*SDHA* c.91C>T p.Arg31* *VHL*** c.589G>A p.Asp197Asn *AIP, MEN1* and *CDKN1B* are not available		PCC	20
3	49/F	Pituitary macroprolactinoma	*SDHA* c.91C>T p.Arg31*	p.D38V; somatic mutation as a second hit of biallelic inactivation	Bilateral HN PGL	present study
4	53/M	Pituitary nonfunctioning macroadenoma	*SDHA* variant c.969C>T p.Gly323Gly†	ROH	Abdominal PGL, Wilms tumor, retroperitoneal liposarcomas & renal oncocytoma	20
5	84/M	Pituitary GH-secreting macroadenoma	*SDHAF2* variant c.-52T>C		HN PGL	20
6	33/M¶	Pituitary macroprolactinoma	*SDHB* c.298T>C p.Ser100Pro	LOH	HN PGL	20
7	36/F¶	Pituitary macroprolactinoma	*SDHB* c.298T>C p.Ser100Pro		BAH	20
8	53/F	Pituitary nonfunctioning macroadenoma	*SDHB* c.587G>A p.Cys196Tyr	LOH	HN PGL	20
9	31/F	Pituitary macroprolactinoma	*SDHB* del ex 6 to 8	LOH		20
10	60/F	Pituitary macroprolactinoma	*SDHB* c.423+1G>A		HN PGL	20

Table 2. Germline *SDH* mutations/variants and pituitary adenomas reported in the literature *(Continued)*

Case	Age^/sex	Functional classification of PA	Germline *SDH* mutation	Biallelic SDH inactivation in PA	PGL/PCC	Reference
11	15/NA	Pituitary adenoma NA	*SDHB* c.761insC p.254fsX255		None	23
12	71/M#	GH-secreting adenoma	*SDHB* exon7 c.689G>A p.Arg230His		Bilateral HNPGL	26
13	51/F#	Pituitary microadenoma	*SDHB* exon 6 c.642+1G>A		Metastatic PGL GIST	26
14	60/M	Pituitary macoprolactinoma	*SDHC* c.256-257insTTT p.Phe85dup		HN PGL	18
15	53/M	Pituitary macroprolactinoma	*SDHC* c.380A>G p.His127Arg		HN PGLs	20
16	36/F	Pituitary macroprolactinoma	*SDHD* c.242C>T p.Pro81Leu		HN PGLs	24
17	41/M	Pituitary GH-secreting macroadenoma	*SDHD* c.298_301delACTC p.T100fsX133	LOH	HN- & ea-PGLs Bilateral PCCs	17
18	56/F	Pituitary GH-secreting macroadenoma	*SDHD* c.274G>T p.Asp92Tyr	ROH	HN PGLs	12 and present study
19	60/M	Pituitary macroprolactinoma	*SDHD* c.274G>T p.Asp92Tyr	LOH	HN PGLs PCC	12 and present study
20	NA	GH-secreting macroadenoma PGL1 syndrome	*SDHD* c.341A>G p.Tyr114Cys			25
21	NA	Pituitary nonfunctioning microadenoma PGL-1 syndrome	*SDHD* c.341A>G p.Tyr114Cys			25
22	NA	pituitary nonfunctioning microadenoma PGL-1 syndrome	*SDHD* c.341A>G p.Tyr114Cys			25
23	NA	Pituitary nonfunctioning microadenoma PGL-1 syndrome	*SDHD* c.341A>G p.Tyr114Cys			25

Table 2. Germline *SDH* mutations/variants and pituitary adenomas reported in the literature *(Continued)*

Case	Age^/sex	Functional classification of PA	Germline *SDH* mutation	Biallelic SDH inactivation in PA	PGL/PCC	Reference
24	40/F#	Pituitary macroprolactinoma	*SDHD* exon3 c.242C>T p.Pro81Leu		Bilateral PCC	26
25	51/F	Pituitary nonfunctioning macroadenoma	*SDHD* Asp92Tyr	NA, radiologic follow-up	HN PGL	present study***

Abbreviations: *SDH* succinate dehydrogenase; *PA* pituitary adenoma; *PGL* paraganglioma; *PCC* pheochromocytoma; *M* male; *F* female; *VHL* Von Hippel-Lindau; *MEN 1* multiple endocrine neoplasia type 1; *AIP* aryl hydrocarbon receptor interacting protein; *CDKN1B* cyclin-dependant kinase inhibitor 1B; *LOH* loss of heterozygosity; *ROH* retention of heterozygosity; *dup* duplication; *HN PGL* head and neck paraganglioma; *BAH* bilateral adrenal hyperplasia; *GH* growth hormone; *NA* not available; *ea-PGL* extra-adrenal paraganglioma; *fs* frame-shift; *GIST* gastrointestinal stromal tumor.

^ Age at diagnosis of the pituitary adenoma.

Age of diagnosis of the new syndromic association.

¶ These patients were first-degree relatives.

** This variant has been described in polycythemia vera but not in classical Von Hippel-Lindau syndrome.

*** One of the excluded patients in the present study, because no biopsy or surgically resected material was available.

† In silico splicing analysis software packages predicted that this variant may create a new splice donor site. RNA was extracted from peripheral blood using PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) but RT-PCR analysis found no evidence of aberrant splicing of the SDHA gene. Sequence analysis of DNA extracted from a paraffin embedded pituitary adenoma sample from this patient showed the presence of this variant with no evidence of loss of the normal allele in the tumor DNA when compared to the peripheral blood DNA. Tissue extracted from the father's nonfunctioning pituitary adenoma (NFPA) did not harbor the variant, while it was present in the germline DNA of the mother, suggesting that it is not the cause of NFPA in father and son. Its role in the proband's other tumors is unknown.

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Supplemental tables and figures

Supplemental Table 1. Clinicopathological and molecular genetic characteristics of nonparaganglionic tumors arising in *SDH* mutation carriers

Case	Age[a]/sex	Germline *SDH* mutation	Tumors observed (age at detection)	SDHB IHC	SDHA IHC	Second inactivation hit ¶	Status at last follow-up (age)
1	56/M	*SDHD* p.Asp92Tyr c.274G>T	pNET (56) Oligodendroglioma (57) GCT (L+R) (56) GVT (R) (56)	Neg Pos	Pos Pos	LOH#	Died (64)
2	61/M[b]	*SDHD* p.Asp92Tyr c.274G>T	Macro-prolactinoma (61) PCC (R) (61) GJTT (R) (60) GCT (L) (60) GVT (L+R) (60)	Neg	Pos	LOH	AWED (69)
3	38/F[c]	*SDHD* p.Pro81Leu c.242C>T	Breast cancer (38) GCT (L) (38)	Hetero-genous^	Pos	ROH	Died due to breast cancer (41)
4	55/F	*SDHD* p.Asp92Tyr c.274G>T	Gastric GIST (55) GCT (L+R) (50) GVT (L) (50)	Neg	Pos	LOH#	AWED (64)
5	42/M	*SDHB* c.423+1G>A	Abdominal ganglioneuroma (42) GVT (R) (42)	Neg	Pos	ROH	AWED (50)
6	45/M[d]	*SDHB* c.423+1G>A	RCC L foci 1 (45) RCC L foci 2 (45) RCC R (45) Gastric GIST (45) No PGL	Neg Neg Neg Neg	Pos Pos Pos Pos	LOH LOH LOH LOH	AWED (47)
7	49/F	*SDHA* p.Arg31X c.91C>T	Macroprolactinoma (49) GCT (R) (26) GCT (L) (49) Meningiomas (49)	Neg Pos	Neg Pos	p.D38V	AWD (meningi-omas) (65)
8	56/F[b]	*SDHD* p.Asp92Tyr c.274G>T	Pituitary adenoma, GH producing (56) GCT (L+R) (56) GJT (R) (56) GVT (R) (56)	Pos	Pos	ROH	Died (71)
9	70/M	*SDHD* p.Asp113fs c.337_340delGACT	Bladder cancer (70) Basal cell carcinoma (71) GCT (L+R) (NA) GJT (R) (NA)	Pos Pos	Pos Pos		Died (73)
10	48/F[e]	*SDHD* p.Asp92Tyr c.274G>T	Endometrial cancer (48) GCT (L+R) (45) GJT	Pos	Pos		AWED (59)

Supplemental Table 1. Clinicopathological and molecular genetic characteristics of nonparaganglionic tumors arising in *SDH* mutation carriers *(Continued)*

Case	Age[a]/sex	Germline *SDH* mutation	Tumors observed (age at detection)	SDHB IHC	SDHA IHC	Second inactivation hit ¶	Status at last follow-up (age)
11	40/F	*SDHD* p.Asp92Tyr c.274G>T	Breast cancer (40) GJT (R) (40) GVT (L) (51) Hodgkin lymphoma (18)	Pos	Pos		AWED (55)
12	57/M	*SDHD* p.Leu139Pro c.416T>C	Melanoma (57)	Pos	Pos		AWED (71)
13	57/F	*SDHD* p.Asp92Tyr c.274G>T	Breastcancer GCT (L+R) (65)	Pos	Pos		AWED (71)
14	57/M	*SDHD* p.Asp92Tyr c.274G>T	Melanoma shoulder (57) PCC (R) (55) GCT (L+R) (NA)	Pos	Pos		AWED (61)
15	68/M	*SDHD* p.Asp92Tyr c.274G>T	Prostate cancer (68) GJTT (L) (57) Abdominal PGL (65)	Pos	Pos		Died due to prostate cancer (71)
16	37/M	*SDHD* p.Asp92Tyr c.274g>T	Testicular cancer (37) Prostate cancer (53) GCT (L+R) (47) GJT (L) (47) GVT (L+R) (48)	Pos Pos	Pos Pos		AWED (62)
17	37/F	*SDHD* p.Asp92Tyr c.274G>T	Papillary thyroid carcinoma (37) GCT (L+R) (37) GVT (R) (37)	Pos	Pos		AWED (63)
18	56/M	*SDHD* p.Asp92Tyr c.274G>T	Bladder cancer (56) GCT (L) (38) GJTT (L) (38) GVT (R) (38)	Pos	Pos		AWD (63)
19	35/F	*SDHD* p.Leu139Pro c.416T>C	Breast cancer (R) (35) Breast cancer (L) (47) GCT (L) (41) GVT (R) (42)	Pos Pos	Pos Pos		AWED (49)
20	64/F	*SDHD* p.Asp92Tyr c.274G>T	Meningioma (64) Mediastinal PGL (67)	Pos	Pos		Died due to malignant PGL (74)
21	65/M	*SDHD* p.Leu139Pro c.416T>C	Prostate cancer (65) Gastric GIST (41) GCT (L+R) (57) GVT (L+R) (57) PCC (L) (62)	Pos NA	Pos NA		AWED (70)
22	50/F	*SDHD* p.Asp92Tyr c.274G>T	Meningeoma (50) Breast cancer (45) No PGL	Pos Pos	Pos Pos		AWED (55)

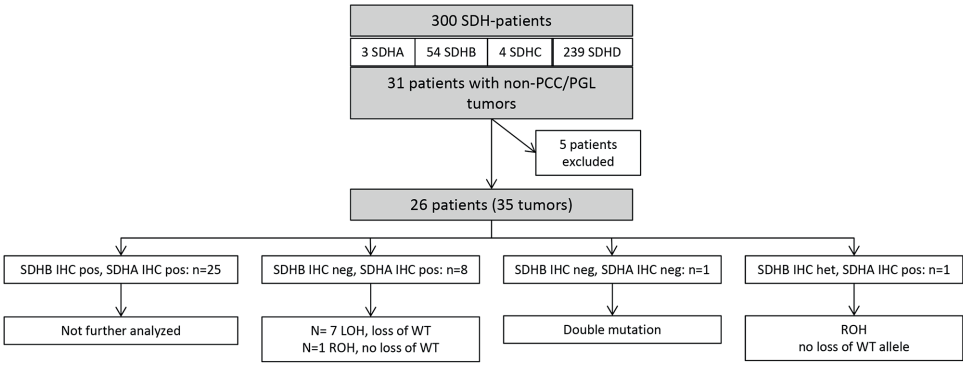
Supplemental Table 1. Clinicopathological and molecular genetic characteristics of nonparaganglionic tumors arising in *SDH* mutation carriers *(Continued)*

Case	Age[a]/sex	Germline *SDH* mutation	Tumors observed (age at detection)	SDHB IHC	SDHA IHC	Second inactivation hit ¶	Status at last follow-up (age)
23	46/F	*SDHB* c.423+1G>A	Basal cell carcinoma (46) No PGL	Pos	Pos		AWED (50)
24	33/F	*SDHB* c.423+1G>A	Breast cancer (33) PGL NA	Pos	Pos		Died due to breast cancer (49)
25	26/F	*SDHB* c.423+1G>A	Melanoma (26) No PGL	Pos	Pos		AWED (49)
26	48/M	*SDHB* p.Thr110Pro unclassified variant c.328A>C	Sebaceous gland carcinoma of the eyelid (48) No PGL	Pos	Pos		AWED (67)

Abbreviations: *M* male; *F* female; *IHC* immunohistochemistry; *pNET* pancreatic neuroendocrine tumor; *GCT* glomus caroticum tumor; *GVT* glomus vagale tumor; *GJT* glomus jugulare tumor; *GJTT* glomus jugulotympanicum tumor; *GTT* glomus tympanicum tumor; *PGL* paraganglioma; *PCC* pheochromocytoma; *RCC* renal cell carcinoma; *GIST* gastrointestinal stromal tumor; *L* left; *R* right; *GH* growth hormone; *AWD* alive with (non-paraganglionic) disease; *AWED* alive without evidence of disease other than head and neck PGL; *NA* not available; *ROH* retention of heterozygosity; *LOH* loss of heterozygosity; *LOH#* only one marker (centromeric or telomeric) was informative in each tumor as indicative of LOH. Sanger (direct) sequencing showed loss of the wild-type allele.

a Age at diagnosis of non-PCC/PGL tumor.
b Patient previously described by Papathomas *et al.*[12]
c Patient also carrier of a germline breast cancer 1 (*BRCA1)* mutation.
d Patient previously described by Gill A *et al.*[16]
e Also heterozygosity for mutation 467C>G, S156X (MSH-6).

¶ Loss of wild-type allele or somatic mutation.
^ Heterogenous is defined as granular cytoplasmic staining combined with a cytoplasmic blush lacking definite granularity or completely absent staining in the presence of an internal positive control throughout the same slide.



Supplemental Figure 1. Overview of the immunohistochemical and sequencing results from 35 nonparaganglionic tumors arising in 26 *SDH* mutation carriers.

Abbreviations: *SDH* succinate dehydrogenase; *PCC* pheochromocytoma; *PGL* paraganglioma; *IHC* immunohistochemistry; *pos* positive; *neg* negative; *het* heterogenous, *LOH* loss of heterozygosity; *ROH* retention of heterozygosity; *WT* wild-type.