This chapter describes the contribution of germline pathogenic *SDHA* variants in paraganglioma patients, assess the clinical manifestations and determine the age-related penetrance.

Clinical Aspects of *SDHA*-Related Pheochromocytoma and Paraganglioma: A Nationwide Study

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ABSTRACT

Context

Paraganglioma (PGL) has the highest degree of heritability among human neoplasms. Current clinical understanding of germline *SDHA* mutation carriers is limited.

Objective

To estimate the contribution of *SDHA* mutations in PGL and to assess clinical manifestations and age-related penetrance.

Design

Nationwide retrospective cohort study.

Setting

Tertiary referral centers in the Netherlands (multicenter).

Patients

Germline *SDHA* analysis was performed in 393 patients with genetically unexplained PGL. Subsequently 30 index *SDHA* mutation carriers and 56 non-index carriers were studied.

Mean outcome

SDHA mutation detection yield, clinical manifestations and *SDHA*-related disease penetrance.

Results

Pathogenic germline *SDHA* variants were identified in 30 of the 393 referred patients (7.6%) with PGL, who had head and neck PGL (21 of 174 [12%]), pheochromocytoma (4 of 191 [2%]) or sympathetic PGL (5 of 28 [18%]). The median age at diagnosis was 43 years (range, 17 to 81 years) in index *SDHA* mutation carriers compared with 52 years (range, 7 to 90 years) in nonmutation carriers (P=0.002). The estimated penetrance of any *SDHA*-related manifestation was 10% at age 70 years (95% confidence interval 0% to 21%) in non-index mutation carriers.

Conclusion

Germline *SDHA* mutations are relatively common (7.6%) in patients with genetically unexplained PGL. Most index patients presented with apparently sporadic PGL. In this *SDHA* series, the largest assembled so far, we found the lowest penetrance of all major PGL predisposition genes. This suggests that recommendations for genetic counseling of at-risk relatives and stringency of surveillance for *SDHA* mutation carriers might need to be reassessed. *(J Clin Endocrinol Metab 103: 438–445, 2018)*

INTRODUCTION

Paragangliomas (PGLs) are rare neuroendocrine tumors that carry the highest degree of heritability among human neoplasms.[1,2] PGLs are classified according to their anatomic location (intra-adrenal or extra-adrenal PGL) and whether they are of sympathetic or parasympathetic origin. Head and neck paragangliomas (HNPGLs) emerge from the parasympathetic nervous system and are usually benign slow-growing tumors.[3,4] Common sites include the carotid body, the temporal bone, and the vagal body. Parasympathetic PGLs are most often nonsecreting, although about 30% are associated with elevated levels of the dopamine metabolite 3-methoxytyramine (3-MT).[5] Pheochromocytoma (PHEO) and sympathetic paraganglioma (SPGL) are catecholamine-secreting tumors.[6] PHEOs are derived from the chromaffin cells of the adrenal medulla and SPGLs are found in close relationship to the peripheral sympathetic nervous system from the level of the superior cervical ganglion down the trunk into the pelvis.[7] Metastases are more often present in SPGL than are to PHEO and HNPGL.[3]

About one third of patients with PGL have been reported to carry pathogenic germline variants in a growing list of susceptibility genes.[8] The most described genes are *NF1, RET, VHL, SDHD, SDHC, SDHB, SDHAF2, SDHA, TMEM127 and MAX*. Germline variants in the succinate dehydrogenase (*SDH*) genes are the most common genetic cause of PGLs, occurring in up to 15% of all patients with PGL and half of all familiar cases.[2,9] In 2010, a direct association between germline *SDHA* variants and PGL was reported.[10] *SDH*-associated syndromes are characterized by the development of PGLs, with an additional risk of developing other tumor types [e.g., clear cell renal cancer (RCC), gastrointestinal stromal tumors (GISTs) and more rarely, neuroendocrine tumors and pituitary adenomas].[11-13] So far, information on prevalence, phenotype, penetrance and pathogenicity of *SDHA* variants is limited to one large series[14] and a few small series.[15,16] In this study we performed a nationwide evaluation of germline *SDHA* analyses undertaken in patients with PGL and characterized the clinical manifestations and disease penetrance in 30 index *SDHA* mutation carriers and their relatives.

PATIENTS AND METHODS

Study population and design

All patients with an established diagnosis of PGL who were referred for germline *SDHA* analysis in the Netherlands from February 2011 through July 2016 were included in this study. Referred patients with PGL were grouped into three clinical subgroups - HNPGL, PHEO, or SPGL - on the basis of clinical, biochemical, imaging and/or histological characteristics. Data on sex, diagnosis and age at diagnosis were retrieved from DNA request forms. In accordance with the Dutch national genetic testing strategy, all patients with PGL referred for *SDHA* analysis lacked pathogenic germline variants in *SDHB*, *SDHC*, *SDHD*, and *SDHAF2*. All patients with PHEO and SPGL furthermore lacked pathogenic germline variants in *TMEM127*, *MAX*, *RET* and *VHL* and had no clinical symptoms suggesting neurofibromatosis type 1.

Index patients with (likely) pathogenic *SDHA* variants or variants of uncertain significance (VUS) were evaluated and subsequently counseled by a clinical geneticist in their regional University Medical Center. Patients with pathogenic and likely pathogenic variants are annotated as *SDHA* mutation carriers in this manuscript. Clinical characteristics (e.g. sex, age at diagnosis, tumor location, or locations, presence of metastases, biochemical phenotype and additional non-PGL tumors) and pedigrees were collected.

Genetic counseling and testing for the family-specific (likely) pathogenic *SDHA* variant were offered to relatives via cascade screening. All *SDHA* mutation carriers age ≥18 years were referred to departments of otorhinolaryngology and endocrinology for annual clinical surveillance aimed at detecting PGL. According to national guidelines,[17] surveillance consisted of magnetic resonance imaging of the head and neck region once every 3 years and magnetic resonance imaging or computed tomography of the thorax, abdomen and pelvis once every 2 years. Annual routine biochemical testing included the measurement of (nor)epinephrine, (nor)metanephrine, dopamine and/or 3-MT in 24 hour urine samples and/or plasma, depending on the center. In cases with excessive catecholamine secretion (i.e., any value above the upper reference limit), radiological assessment of the thorax, abdomen, and pelvis was performed to identify potential sources of excessive catecholamine production. The current study was approved by the local medical ethical committee of Leiden University Medical Center (G16.063).

DNA sequencing and data analysis

Germline *SDHA* variant analysis was performed in the d\Department of Human Genetics at the Radboud University Medical Center and the Laboratory for Diagnostic Genome Analysis of the Department of Clinical Genetics at Leiden University Medical Center, the Netherlands. Genomic DNA was extracted from peripheral blood leukocytes according to standard procedures. Germline *SDHA* analysis was performed with Sanger sequencing or next generation (gene panel) sequencing (NGS) depending on the testing period. For the detailed NGS procedure, see the Supplemental Method.

Coding variants were analyzed for their effect on function by using the Alamut software package version 2.7 (Interactive Biosoftware, Rouen, France), which incorporates Align GVGD,[18] polymorphism phenotyping (PolyPhen2),[19] and sorting intolerant from tolerant (SIFT).[20] Variants were annotated to the Genbank reference sequence NM_004168.2. The Leiden Open Variation Database (http://www.lovd.nl/*SDHA*) was consulted to find variants previously described and classified. Variant interpretation was done in line with the recent consensus statement on NGS-based diagnostic testing of PHEO and PGLSs.[21] Variant nomenclature is in accordance with Human Genome Variation Society guidelines version 2.0 (http://www.hgvs.org).

To obtain further support for the pathogenicity of certain *SDHA* variants, SDHA immunohistochemistry and loss-of-heterozygosity (LOH) analysis were performed on formalin-fixed, paraffin-embedded samples, as described elsewhere.[22]

Statistical analysis

Descriptive statistics were used to characterize the study population, to determine the age of PGL onset, and to examine the difference between patients with germline *SDHA* mutation and those without germline *SDHA* mutation. Continuous variables were analyzed by using an independent sample t test. Dichotomous variables were compared using the χ^2 test. Age-related penetrance of PGL was estimated by using the Kaplan-Meier method. Because most of the non-index mutation carriers were recently identified, we used the age at 1 year after DNA analysis (or age at death) for the penetrance estimation. By completion of this manuscript, 80% of the non-index mutation carriers had participated in surveillance at least once. Statistical significance was set at $P < 0.05$, and the analyses were conducted by using SPSS software, version 23.0 (IBM, Armonk, NY).

RESULTS

SDHA case detection in the study population

Pathogenic germline *SDHA* variants were identified in 30 of 393 (7.6%) patients with PGL who were referred for *SDHA* genetic testing. The clinical characteristics of the study population *(SDHA* vs. non-*SDHA)* are listed in Table 1. Within the clinical PGL subgroups, pathogenic *SDHA* variants were identified in 21 of 174 patients with HNPGL (12%), 4 of 191 patients with PHEO (2%), and 5 of 28 patients with SPGL (18%). The median age at diagnosis of PGL was 43 years (range, 17 to 81 years) in *SDHA* mutation carriers and 52 years (range, 7 to 90 years) in those without a detectable mutation (p=0.002). Half of the *SDHA* mutation carriers were males, compared with to 32% of patients without an *SDHA* mutation (P = 0.049).

SDHA variants

Seven different (likely) pathogenic germline *SDHA* variants were identified in 30 patients with PGL (Table 2). Three variants had been reported previously: The common nonsense mutation c.91C>T, (p.Arg31*)[10] was observed in 23 patients, the c.1753C>T, (p.Arg585Trp)[16] missense mutation was observed in two patients and the nonsense c.1534C>T, p.(Arg512*)[22] was observed in one patient. Moreover, four not previously reported and three previously reported *SDHA* VUS were identified (Supplemental Table 1). All VUS were identified in patients with apparently sporadic HNPGL diagnosed between the ages of 28 and 52 years. Additional immunohistochemical staining and LOH analysis were performed in two of the seven VUS-related HNPGLs, but showed no loss of SDHA or SDHB staining or LOH. The other five HNPGLs were not available for further analysis.

Table 1. Clinical Characteristics of Patients With Germline SDHA Mutation and Patients Without Germline *SDHA* Mutation

Characteristic	*SDHA* mutation (n=30)	No *SDHA* mutation (n=363)	*P*-value	Mutation Yield (%)
All patients with PGL				7.6
Age diagnosis (y)	43 (17–81)	52 (7–90)	0.002	
Males, n (%)	15 (50)	117 (32)	0.049	
HNPGL	21	153		12
Age diagnosis (y)	43 (18-81)	54 (19-90)	0.008	
Males, n (%)	10 (48)	33 (22)	0.010	
PHEO	4	186		2
Age diagnosis (y)	35 (17-70)	51 (7-81)	0.14	
Males, n (%)	0	71 (38)	0.118	
SPGL	5	24		18
Age diagnosis (y)	36 (22-60)	53 (23-73)	0.118	
Males, n (%)	5 (100)	13 (54)	0.055	

Data are presented as median (range) or number and percentage. *P* values are derived from χ^2 or independent sample t test.

Table 2. Clinical and Molecular Characteristics of 30 Index SDHA Mutation Carriers and Their Relatives

Patient No.	Family	Sex	Tumors Observed (Age at Detection, y)	Biochemistry Results
1	A	F	GCT-ri (43)	Normal
2	B	M	GVT-le (38)	Normal
3	C	F	GVT-ri (81)	Normal
4	D	F	GCT-le 2x (35)	Normal
5	E	F	GCT-le, GCT-re, GJT-le (48)	Normal
6	F	M	GCT-le (30)	NA
7	G	M	GCT-ri (56), Prolactinoma (58)	Normal
8	H	M	GJT-le (43)	Normal
9	I	F	GCT-ri, GCT-le (26, 49, 50), Prolactinoma (45), Multiple Meningioma (45,62)	Normal
10	J	M	GCT-ri (23)	Normal
11	K	M	GJTT-le (38)	3-MT
12	L	F	GCT-le (40)	Normal
13	M	F	GCT-le (61)	NA
14		F	GJTT-ri (58)	Normal
15		F	GVT-le (53)	Normal
16		F	GVT-ri (42)	Normal
17		F	GVT-ri (53)	Normal
18		M	GJT-ri (18)	NA
19		M	GCT-ri (48), Uveal melanoma (48)	Normal
20		M	GVT-ri (60)	3-MT
21		M	GVT-ri, GVT-le, GCT-ri (49)	Normal
22	N	F	PHEO-ri (17)	NM
23		F	PHEO-ri (20), Wilms tumor (4)	NM
24		F	PHEO-le (50), metastasis (60)	NM
25		F	PHEO-ri (70), RCC (70)	Normal
26	O	M	Para-aortal SPGL (60)	NA
27	P	M	Testis SPGL (23), metastases (26)	NM/3-MT
28	Q	M	Retroperitoneal para-aortal SPGL (50)	NM/3-MT
29	R	M	Malignant retroperitoneal SPGL (36)[f]	NM/3-MT
30	S	M	Para-aortal SPGL (22)	NM

See Supplemental Fig. 2 for the pedigrees of families A through S.
Abbreviations: GCT; glomus carotid body tumor, GJT; glomus jugularis tumor, GJTT; glomus jugulotympanicum tumor, GTT; glomus tympanicum tumor, GVT; glomus vagal tumor, ri; right, le; left, NA; not available, PTC;

Family History	Germline *SDHA* variant	Tested relatives (Carriers[a])	Reference
Negative	c.91C>T, p.(Arg31*)	5 (3)	[10]
Negative	c.91C>T, p.(Arg31*)	2 (1)	[10]
Negative	c.91C>T, p.(Arg31*)	3 (1)	[10]
Negative	c.91C>T, p.(Arg31*)	1 (1)	[10]
Negative	c.91C>T, p.(Arg31*)	6 (4)	[10]
Negative	c.91C>T, p.(Arg31*)	10 (6)	[10]
Negative	c.91C>T, p.(Arg31*)	4 (2)	[10]
Negative	c.1432_1432+1delGG[b]	3 (2)	Novel
Negative	c.91C>T, p.(Arg31*)	8 (2)	[10]
GIST[c]	c.91C>T, p.(Arg31*)	8 (6)	[10]
Negative	c.91C>T, p.(Arg31*)	2 (1)	[10]
RCC[c]	c.985C>T, p.(Arg329*)	1 (1)	Novel
Negative	c.91C>T, p.(Arg31*)	8 (5)	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
RCC[d]	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.1795-3C>G[¥]	0	Novel
Negative	c.667delG, p.(Asp223fs)	0	Novel
Negative	c.1753C>T, p.(Arg585Trp)	3 (1)	[16]
Negative	c.1753C>T, (p.Arg585Trp)	0	[16]
Negative	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
RCC[e]	c.91C>T, p.(Arg31*)	10 (8)	[10]
HNPGL[c]	c.1534C>T, p.(Arg512*)	3 (4)	[22]
Negative	c.91C>T, p.(Arg31*)	8 (3)	[10]
Negative	c.91C>T, p.(Arg31*)	8 (2)	[10]
Negative	c.91C>T, p.(Arg31*)	3 (3)	[10]

papillary thyroid carcinoma, [a] Including obligate mutation carriers, [b] Likely pathogenic *SDHA* variant, [c] *SDHA* mutation carrier, [d] *SDHA* mutation status unknown, [e] No *SDHA* mutation carrier, [f] Dead of disease. Reference sequence *SDHA:* NC000005.9, NM004168.3

Clinical manifestations in index *SDHA* mutation carriers

The clinical and molecular characteristics of the 30 index patients with germline pathogenic *SDHA* variants are listed in Table 2. Germline *SDHA* mutations were identified in 21 index patients with HNPGL, 4 with PHEO and 5 with SPGL. Four patients were diagnosed with multiple HNPGLs. The HNPGL anatomic locations were distributed as follows: 15 carotid body, 8 vagal, 3 jugular, and 2 jugular tympanic. Two patients with HNPGL had elevated 3-MT levels. Three patients with PHEO had elevated normetanephrine levels. One patient had developed a metastatic PHEO, but no bilateral PHEOs were detected. Four SPGLs had a retroperitoneal para-aortal location and one SPGL was found in the testis. Four patients with SPGL had elevated normetanephrine levels, three in combination with elevated 3-MT.Two patients developed metastatic SPGL and one of these patients (no. 27) died at age 27 years.

Furthermore, three index mutation carriers (no. 7, no. 9, no. 25) were diagnosed with one other possibly *SDHA*-related feature, including pituitary adenoma (at ages 58 and 45 years, respectively) and RCC (age 70 years), respectively. One pituitary adenoma was immunonegative for both *SDHA* and *SDHB* and contained an additional somatic pathogenic *SDHA* variant (p.Asp38Val), likely resulting in biallelic inactivation of *SDHA* (Supplemental Fig. 1).[23] The other pituitary adenoma was not resected and therefore not analyzed. Conversely, the RCC tissue showed no loss of SDHA immunohistochemical staining, suggesting that it was not *SDHA*-related. Three additional tumor types were reported in index *SDHA* mutation carriers: multiple meningioma (patient no. 9), uveal melanoma (no. 19, *BAP1*-mutation negative) and Wilms tumor (no. 23). However, it is not clear whether these tumors were related to the *SDHA* mutation. Immunohistochemical staining showed no loss of SDHA staining in both meningiomas. The uveal melanoma lesion and Wilms tumor were not available for analysis. Five *SDHA* mutation carriers had a positive family history for *SDHA*-related tumors, including HNPGL (patient no. 27), GIST (no. 10), and RCC (no. 12, no. 17 and no. 26).

Clinical manifestations in *SDHA* families

In total, 94 available relatives were tested via cascade screening for their familial pathogenic *SDHA* variant, revealing 51 non-index carriers and 5 obligate carriers. Pedigrees of the 19 *SDHA* families

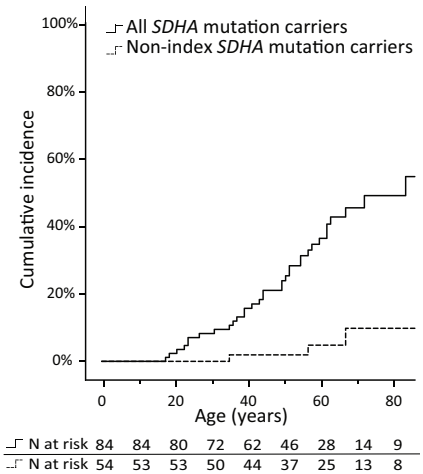

Figure 1. Age related penetrance of any *SDHA*-related manifestations

with at least one non-index mutation carrier are shown in Supplemental Fig. 2. Remarkably, we could confirm in all families, except one (index no. 3 diagnosed at age 81 years), that the mutation was inherited from an unaffected parent. The median age at DNA analysis in the non-index *SDHA* mutation carriers was 58 years (range, 7 to 94 years). In total, 3 of 56 (5%) non-index *SDHA* mutation carriers were diagnosed as having one (possible) *SDHA*-related tumor: HNPGL (n=1), GIST (n=1), and RCC (n=1). Family history did not reveal any not-tested relatives with (possible) *SDHA*-related tumors.

The estimated penetrance of any *SDHA*-related tumor is shown in Fig. 1. The age-related penetrance values for all 86 *SDHA* mutation carriers were 7% at age 25 years [95% confidence interval (CI) 2% to 12%], 26% at age 50 years (95% CI 16% to 36%) and 50% at age 70 years (95% CI 34% to 66%). The age-related penetrance values for the 56 non-index *SDHA* mutation carriers were 0% at age 25 years, 2% at age 50 years (95% CI 0% to 6%) and 10% at age 70 years (95% CI 0% to 21%). By completion of this manuscript, 51 non-index carriers were lacking any identified *SDHA*-related feature, indicating that they could be considered to be healthy mutation carriers.

DISCUSSION

This nationwide retrospective *SDHA* survey investigated *SDHA* mutation detection yield and clinical phenotype in patients with genetically unexplained PGL. We identified pathogenic germline *SDHA* variants in 30 of 393 (7.6%) patients with PGL. Most of our index *SDHA* mutation carriers presented with an apparently sporadic HNPGL. Remarkably, most germline mutations were inherited from an unaffected parent. The clinical phenotype in our *SDHA* families is similar to that seen in previous studies (i.e., with few non-PGL tumors, such as GIST, RCC, and pituitary adenoma).[2,14] This study highlights the low age-related penetrance: 10% at age 70 years in non-index *SDHA* mutation carriers. However, some index mutation carriers presented at very young ages and/or with metastatic disease. These results may give cause to reconsider the current surveillance protocol for *SDHA* mutation carriers. The age at first examination and/or the interval between screenings could possibly be less stringent than for *SDHB/C/D* mutation carriers.

SDHA mutation analysis

On the basis of a detection yield of 7.6% in this nationwide cohort analysis, we recommend germline *SDHA* analysis for all individuals with PGL, preferably by using gene panels. To date, at least 15 genes have been associated with hereditary PGL and it is likely that further rare and low-penetrant genes will be identified. Until recently, a step-wise mutation testing protocol was applied in those suspected of having familial PGL. Multiple algorithms were used, including age at presentation, location of tumor, multifocal or metastatic disease, presence of syndromic features and family history.[1] This type of testing protocol is expensive and time-consuming. Nowadays, gene panel testing using NGS is fast and cost-effective for germline genetic testing of patients with PGL.[24] However, molecular analysis of *SDHA* in NGS panels could be challenging because of the presence of four pseudogenes that are highly homologous to both the coding regions of *SDHA* and the intronic regions of the gene. According to our data, additional *SDHA* Sanger sequencing should be considered in patients with HNPGL and SPGL without detectable mutations following NGS. The *SDHA* mutation detection yield in patients with apparently sporadic HNPGL in our study population (12%) was higher than in a previous study (6%), whereas the detection yield in patients with PHEO in our study population (2%) was similar to that of patients without HNPGL in that study (2%).[14] Although no specific data are available on *SDHA* mutation detection yields in SPGLs, our detection yield was high (18%); however, it was seen against a background of a small sample size. We identified eight *SDHA* variants not previously reported, including four pathogenic

variants and four VUS. More than 60 unique *SDHA* nonsense and missense variants have been reported in the Leiden Open Variation Database, and they are evenly distributed across coding exons. No *SDHA* genotype-phenotype relationship has yet been established. Among Dutch *SDHA* index mutation carriers, the pathogenic variant c.91C>T (p.Arg31*) was most frequent (21 of 30), in contrast to 5 of 29 in a previous study of non-Dutch index patients, an over four-fold difference in frequency.[14] This variant has an allele frequency of 0.027% in the Exome Aggregation Consortium database and 0.039% (6:16000) in our in-house whole exome sequencing database (unpublished data). Together, these data suggest that *SDHA* p.Arg31* is a Dutch founder mutation, in the same vein as the very common *SDHB* and *SDHD* founder mutations reported in the Netherlands.[25]

Genetic counseling

Exploring the genetic basis of hereditary PGL after appropriate counseling provides opportunities for early detection of PGL in patients and relatives. Early removal of tumors may prevent or minimize complications related to mass effects, catecholamine hypersecretion, and metastatic transformation. However, this is counterbalanced by the need for lifelong surveillance starting at an early age and the possible psychological burden of not knowing whether, when and how (benign or metastatic) these tumors will develop. This is a particular challenge in the case of *SDHA*, for which penetrance appears to be much lower than for *SDHD* and somewhat lower than for *SDHB*.[26] Prospective studies in *SDHA* mutation carriers - including genotype-phenotype relationships, genetic modifiers and/or environmental factors - are required to determine the optimal age at which surveillance should be initiated and the best monitoring intervals to capture the different *SDHA*-related manifestations as they develop.

Strengths and limitations of the study

The current study has several strengths as well as some limitations. Its main strength is the size of the cohort investigated, representing the largest *SDHA* series to date (n = 84 carriers). This was possible because of the close collaboration of several of Dutch university medical centers. A further strength is that all patients with PGL referred for germline *SDHA* analysis in the Netherlands within a defined period (2011 to 2016) were included in the study. Finally, the study was initiated at the Center for Endocrine Tumors Leiden and the Radboud Adrenal Center, both tertiary referral centers recognized as national and European centers-of-excellence for rare endocrine tumors, including PGL. The study also has a number of limitations. First, the estimated mutation detection yield in this study was found in a retrospective diagnostic cohort and therefore might not be representative of the total patient population. However, a large proportion of the study population was systematically referred within a defined period and these patients did not differ in age, sex and diagnosis from other patients (unpublished data). Second, a possible explanation for the relatively low penetrance in our *SDHA* families could be inadequate surveillance and incomplete follow-up data. On the other hand, the over-representation of index patients (29 of 37), in a previous study leads to an overestimation of penetrance.[14]

Conclusion

Germline *SDHA* mutations are relatively frequent (7.6%) in patients with genetically unexplained PGL, even in the absence of familial or clinical indications for inherited PGL. Mutation analysis of *SDHA* should therefore be included in the genetic testing of all patients with PGL, preferably by using gene panels. This study confirms the long-suspected low penetrance of disease in *SDHA* mutation carriers and suggests that recommended guidelines for genetic counseling of at-risk relatives and surveillance in mutation carriers might need to be revised.

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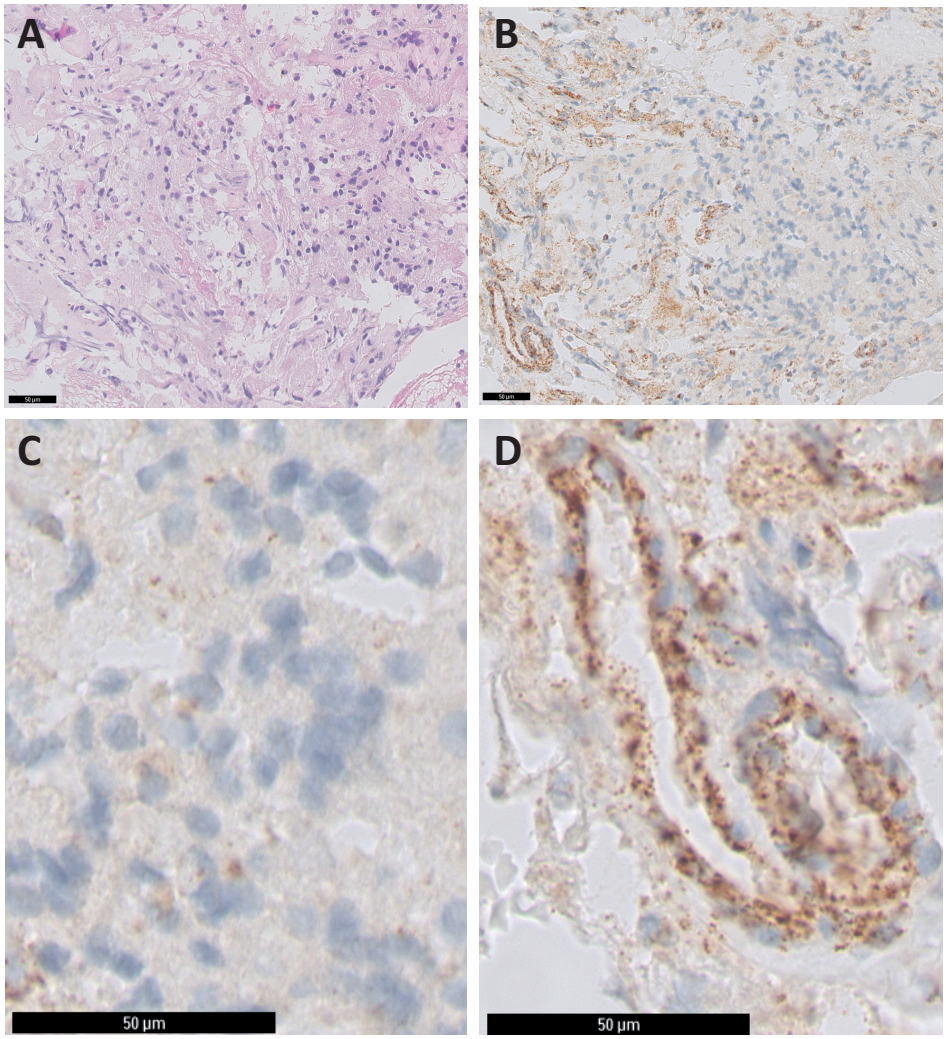
SUPPLEMENTAL DATA

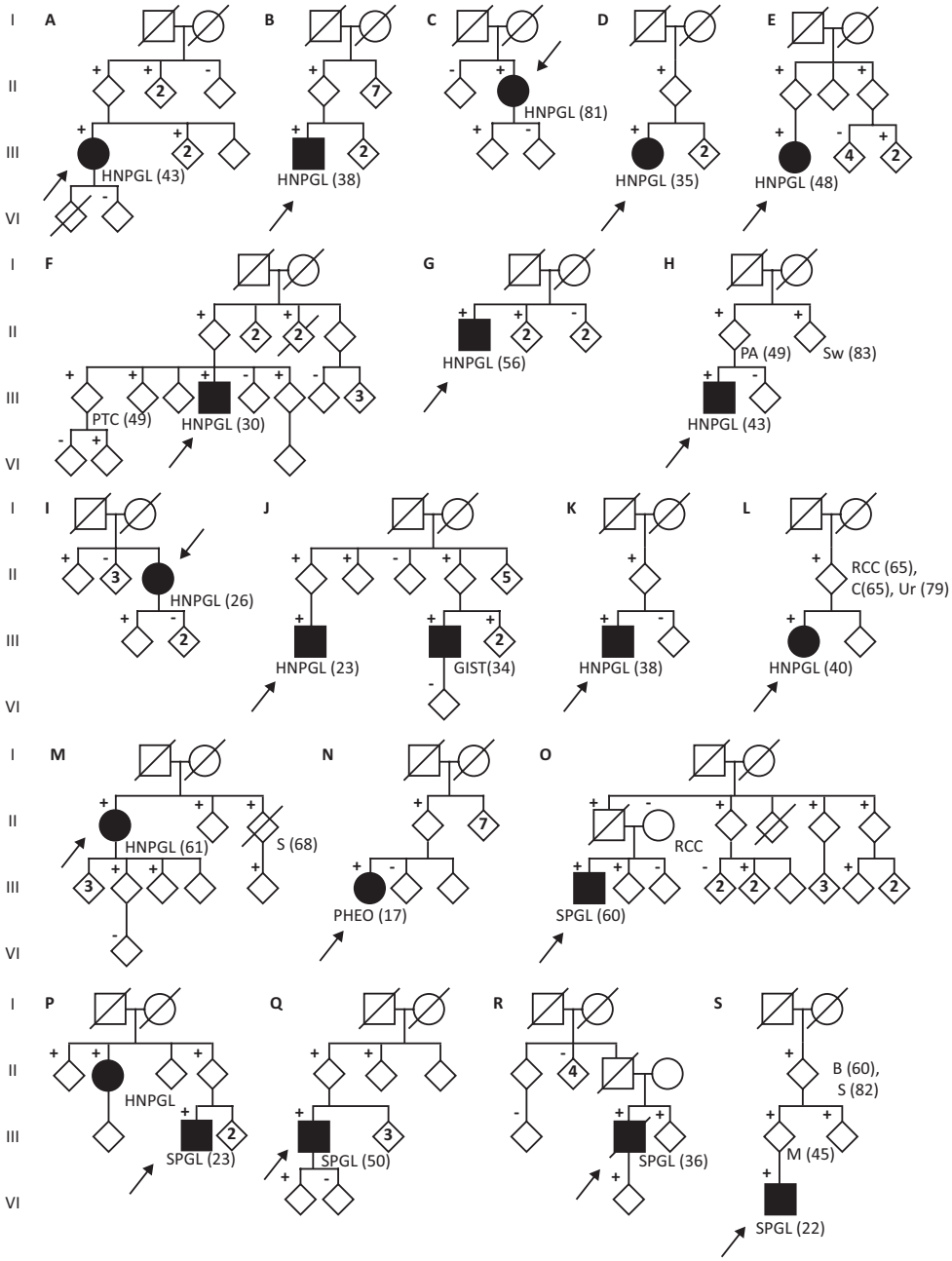
Supplemental Table 1. Overview of the clinical and molecular characteristics of 7 patients with *SDHA* variants of uncertain significance

ID #	Tumors observed (age at detection,y)	Gender	Location tumor	SDHA IHC	Family history	*SDHA* variant	Reference
31	HNPGL (52)	Female	GJT-right	Normal	Negative	c.778G>A, p.(Gly260Arg)	Bausch *et al*. 2017 (class 4), Bannon *et al*. 2017 (loss-of-function)
32	HNPGL (42)	Female	Unknown	Normal	Negative	c.889C>T, p.(Pro297Ser)	novel
33	HNPGL (42)	Male	GCT-left	NA	Negative	c.1115C>G, p.(Pro372Arg)	Bausch *et al*. 2017 (class 3)
34	HNPGL (28)	Female	GJTT-left	NA	Negative	c.1260+1G>A	novel
35	HNPGL (29)	Female	Unknown	NA	NA	c.969C>T, p.(Gly323Gly)	novel
37	HNPGL (33)	Male	GVT-right	NA	Negative	c.1352G>A, p.Arg451His	novel
38	HNPGL (48)	Female	GJT-right	NA	Negative	c.622-8T>C, (p.?)	novel

Abbreviations: HNPGL; head neck paraganglioma, GJT; glomus jugulars tumor, GTT; glomus tympanicum tumor, GJTT; glomus jugulotympanicum tumor, GVT; glomus vagal tumor, GCT; glomus carotid body tumor, IHC; immunohistochemical staining, LOH; loss of heterozygosity wild type *SDHA* allel, NA; not available



Supplemental Figure 1. Immunohistochemical staining SDHA related tumor. ID#9, carriers the germline pathogenic c.91C>T, p.(Arg31*) *SDHA* variant. **A.** Hematoxylin & Eosin (HE) staining prolactinoma, somatic pathogenic p.D38V *SDHA* variant (see Niemeijer *et al*. 2015) **B.** immunohistochemical staining displaying loss of SDHA expression in the **C.** neoplastic cells, with **D.** normal (endothelial) cells serving as positive internal controls. SDHA mouse monoclonal Ab14715 antibody (1:500 dilution; Mitosciences; Abcam)



Supplemental Figure 2. Pedigrees of 19 *SDHA* families. Please note that the pedigree has been adjusted to protect the identity of the family without a loss of scientific integrity. Circles represent females, squares represent males, diamonds represent undisclosed gender, cross striped individuals are deceased, age at diagnosis between brackets, HNPGL; head- neck paraganglioma, PHEO; pheochromocytoma, SPGL; sympathetic paraganglioma, GIST; gastrointestinal stromal tumor, RCC; renal clear cell tumor, B; breast cancer, C; colon cancer, Ut; urothelial cancer, M; melanoma, S; sarcoma, Sw; schwanoma, *SDHA* mutation carrier (+), non-carriers (-).

Supplemental Method

DNA sequencing

Mutation analysis was performed at the Radboud University Medical Center or the Leiden University Medical Center.

For Sanger sequencing, all exons and intron-exon boundaries of *SDHA* were sequenced with M13-labeled primers specifically targeting *SDHA* (NM_004168.2), but not the 4 pseudogenes: *SDHAP1* (ENSG00000185485) LOC220729 (ENSG00000214135) and *SDHAP2* (ENSG00000215837) on chromosome 3q29, and *SDHAP3* (ENSG00000185986) on chromosome 5p15. Primer sequences are available on request.

Next generation panel sequencing included all exons and intron-exon boundaries of *SDHA* (NM_004168.2), *SDHB* (NM_003000.2), *SDHC* (NM_003001.3), *SDHD* (NM_003002.3), *SDHAF2* (NM_017841.2), *VHL (*NM_000551.3), *MAX* (NM_002382.4) and *TMEM127* (NM_017849.3), and exons 10, 11, and 13 to 16 of the *RET* proto-onco gene (NM_020975.4). Ampliseq primer pools were designed using the online designer tool from Life Technologies (http://www.ampliseq.com). No SNPs with a frequency of over 0.5% were allowed in the five most 3' nucleotides, which was confirmed by SNPCheck v3 analysis (https://ngrl.manchester.ac.uk/SNPCheckV3/snpcheck.htm). At most four low-frequency SNPs were allowed at other positions. The coding sequence and at least 10 bp of padding sequence including the intron-exon boundaries was fully covered. In cases where exons were covered by multiple amplicons overlap was ensured, without overlap of primer sequence. The full coding sequences were covered in two multiplex pools consisting of 34 and 28 amplicons, respectively. Primer sequences are available on request.

Ampliseq enrichment and Ion Torrent PGM sequencing

Libraries were generated according to the manufacturer's protocol, using at least 10 ng of DNA per pool and amplification in 19 cycles using the Ion Ampliseq mastermix (Life Technologies). After barcode and adapter ligation DNA was purified with Agencourt AMPure XP beads (Beckman Coulter Genomics, High Wycombe, UK). The library was diluted to 12 pM. Emulsion PCR was performed using the Ion OneTouch 200 Template kit on the Ion OneTouch System (Life Technologies). Next, Ion Sphere Particles (ISPs) were recovered and enriched for template positive ISPs using Dynabeads MyOne Streptavidin C1 beads (Life Technologies) in the Ion OneTouch ES instrument (Life Technologies). ISP enrichment was quantified using a Qubit 2.0 fluorometer (Life Technologies). Sequencing primer and polymerase were added to the final enriched spheres before loading onto a 316 chip using the Ion PGM 200 sequencing kit (Life Technologies). Chips were run on a Ion Torrent Personal Genome Machine (PGM) (Life Technologies).

NGS sequencing data analysis

Data from the PGM runs were processed using Ion Torrent Suite v3.1 to generate sequence reads, trim adapter sequences, filter and split the reads according to the barcode in succession. FastQ files were loaded into JSI Seqpilot module SeqNext v4.0 (JSI Medical Systems, Kippenheim, Germany) for mapping and analysis of the data. Pseudogene reads of *SDHA* and *SDHD* were filtered out by skipping the reads containing at least two pseudogene variants.

All nucleotides were covered at least 40X, or Sanger sequenced if this depth could not be reached (especially exon 1 and 14 of *SDHA*).

Variants were called if the variant frequency was >15% of all reads in both the forward and the reverse direction. All identified (likely) pathogenic variants were confirmed using Sanger sequencing.