Germinal defects of *SDHx* genes in patients with isolated pituitary adenoma

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Abstract

Background: The '3PAS' syndrome, associating pituitary adenoma (PA) and pheochromocytoma/paraganglioma (PPGL), is sometimes associated with mutations in PPGL-predisposing genes, such as *SDHx* or *MAX*. In '3PAS' syndrome, PAs can occur before PPGL, suggesting a new gateway into *SDHx*/*MAX*-related diseases.

Objective: To determine the *SDHx*/*MAX* mutation prevalence in patients with isolated PAs and characterize PAs of patients with *SDHx*/*MAX* mutations.

Design: Genes involved in PAs (*AIP*/*MEN1*/*CDKN1B*) or PPGLs (*SDHx*/*MAX*) were sequenced in patients with isolated PAs. We then conducted a review of cases of PA in the setting of '3PAS' syndrome.

Results: A total of 263 patients were recruited. Seven (likely) pathogenic variants were found in *AIP*, two in *MEN1*, two in *SDHA*, and one in *SDHC*. The prevalence of *SDHx* mutations reached 1.1% (3/263). Of 31 reported patients with PAs harboring *SDHx*/*MAX* mutations (28 published cases and 3 cases reported here), 6/31 (19%) developed PA before PPGL and 8/31 (26%) had isolated PA. The age of onset was later than in patients with *AIP*/*MEN1* mutations. PAs were mainly macroprolactinomas and showed intracytoplasmic vacuoles seen on histopathology.

Conclusions: We discovered *SDHx* mutations in patients bearing PA who had no familial or personal history of PPGL. However, the question of incidental association remains unresolved and data to determine the benefit of *SDHx*/*MAX* screening in these patients are lacking. We recommend that patients with isolated PA should be carefully examined for a family history of PPGLs. A family history of PPGL, as well as the presence of intracytoplasmic vacuoles in PA, requires *SDHx*/*MAX* genetic testing of patients.

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Introduction

Although pituitary adenomas (PAs) are benign tumors, they can be responsible for clinical features due to hormonal disturbances and symptoms of compression that are secondary to local invasion and that may lead to hypopituitarism. The reported prevalence of symptomatic PAs is as high as 1 in 1000 (1). PAs are most frequently sporadic diseases, but are inherited in approximately 5%

of cases. In these cases, PAs may be isolated, as in familial isolated pituitary adenomas (FIPAs) due to *AIP* mutation (*AIP*; OMIM 605555), or occur in a syndromic association, such as (1) multiple endocrine neoplasia 1 (*MEN1*; OMIM 131100), which predisposes patients mainly to primary hyperparathyroidism, endocrine duodeno-pancreatic tumors, and PA; and more rarely, (2) multiple endocrine

neoplasia 4 (MEN4; OMIM 610755), which represents a MEN1-like syndrome; or (3) Carney complex (CNC; OMIM 160980) which has cutaneous manifestations, acromegaly, Cushing syndrome, myxoma and schwannoma (2). In syndromic forms or familial cases, patients may benefit from genetic screening to propose specific monitoring and genetic counseling.

Pheochromocytomas are neuroendocrine tumors arising from adrenal medulla cells. Paragangliomas originate from sympathetic or parasympathetic ganglia. The release of catecholamines by pheochromocytoma/paraganglioma (PPGL) can lead to episodes of hypertension, profuse sweating, headaches, panic attacks, arrhythmia, stroke, and death. The standard treatment is resection and management of PPGL requires highly specialized teams. Around 40% of PPGL are genetically determined (3). Among the 20 or so predisposing genes that have been identified, some lead to a syndromic form of the disease (*VHL*, *NF1*, *RET*, *FH*), while some lead to isolated forms (including *SDHD*, *SDHC*, *SDHB*, *SDHA*, *SDHAF2*, *MAX*, *SLC25A11*, *TMEM127*). Germinal mutations are found in *SDHx* genes (*SDHD*, *SDHC*, *SDHB*, *SDHA* and *SDHAF2*), encoding for the SDH subunits in half of the hereditary cases.

The association between PA and PPGL was first described by Iversen in 1952 (4). This association can occur during MEN1 syndrome or independently (5, 6, 7, 8, 9). This condition, called '3PAs' syndrome (for pituitary adenoma/pheochromocytoma/paraganglioma association) by Xekouki, can be described as the co-occurrence of PA and PPGL without other features of MEN1 syndrome (5, 6, 10). This association is rare, with fewer than 100 cases published in 2019. The '3PAs' syndrome may be associated with germline mutations in genes responsible for predisposition to non-syndromic PPGL, such as genes encoding for SDH subunits or *MAX* (6, 7, 8, 9).

The objective of this study was to assess the involvement of the main non-syndromic PPGL-predisposing genes in patients with isolated PAs and to study the PA characteristics of patients with *SDHx*/*MAX* mutations. To this end (1), we determined the prevalence of germline mutations in *MEN1*, *CDKN1B*, and *AIP* and in *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2* (herein called *SDHx* genes) and *MAX* in a large series of patients for which genetic testing was performed for sporadic or familial isolated PA (2). We reviewed the literature for published cases of PA in the setting of '3PAs' syndrome to determine if patients with PA and genetic mutations in PPGL-predisposing genes show phenotypic differences

compared to patients with *AIP*, *MEN1*, *PRKARIA*, or *CDKN1B* mutation and those with non-genetically determined PAs.

Patients and methods

Subjects

All patients who underwent genetic testing in the context of an isolated PA, without other endocrine lesions, at the molecular laboratory of Marseille Conception Hospital between November 2016 and December 2018 were included. Written informed consent from all patients for genetic analysis was obtained during one-on-one genetic counseling. This study was approved by the ethics committee of Aix-Marseille University (approval number: 2018-13-12-004).

Next-generation sequencing (NGS)

Genomic DNA was extracted from blood lymphocytes (standard EDTA samples) using QiaSymphony DS DNA Midi Kit (Qiagen). Exons and 20 bp flanking introns of *AIP* (NM_003977.2), *MEN1* (NM_130799.2), *CDKN1B* (NM_004064), *SDHA* (NM_004168.2), *SDHB* (NM_003000.2), *SDHC* (NM_003001.3), *SDHD* (NM_003002.2), *SDHAF2* (NM_017841.1) and *MAX* (NM_002382.3) were sequenced by next-generation sequencing (NGS) using the QiaSeq library (Qiagen) according to the manufacturer's instructions. This library included unique molecular indices that are added to each DNA fragment before amplification, in order to reduce the sequencing error rates and to suppress PCR duplicates. Libraries were sequenced on MiSeqDX (Illumina). The alignment and variant calling were performed using the Biomedical Genomics Workbench 5.0.1 (Qiagen) and based on human genome GRCh37/hg19. Annotation was done using VariantStudio v2.2 (Illumina), according to the HGVS guidelines (11). All regions were sequenced with coverage of depth superior to 30x after removing PCR duplicates.

We then performed copy number variation (CNV) analysis using the CNV detection tools from the Biomedical Genomics Workbench, based on the relative depth of coverage of specimen.

Each variant was classified according to the guidelines of the American College of Medical Genetics and Genomics (ACMG) into one of the five following classes (12):

Class 1: benign variant (BV)

Class 2: likely benign variant (LBV)

Class 3: variant of uncertain significance (VUS)

Class 4: likely pathogenic variant (LPV)

Class 5: pathogenic variant (PV)

In silico predictions were performed using Alamut Visual software (Interactive Biosoftware, Rouen, France), including the conservation level, SIFT, PolyPhen-2, and the study of the splicing impact. The population data from population database (gnomAD database, <https://gnomad.broadinstitute.org/>, last visited November 2019) and from inherited disease databases, ClinVar, LOVD, and HGMD, were collected. Variants with a frequency greater than 5% in the population were not retained. All PVs and LPVs were confirmed by Sanger sequencing (primers and protocols are available upon request).

Exploration of SDHx mutations in pituitary adenomas

To examine the role of the SDHx germinal mutation in PAs, both SDHB immunohistochemistry (IHC) and research of loss of heterozygosity (LOH) were performed on formalin-fixed paraffin-embedded (FFPE) slides obtained from surgically removed samples of the pituitary adenoma, where this was available.

SDHB IHC analysis

The investigation of the loss of SDH complex expression in neoplastic cells was performed using a commercially available polyclonal antibody against SDHB (Sigma Aldrich, HPA002868) at a dilution of 1:150. If any component of the SDH complex is damaged, the entire SDH complex becomes unstable, releasing the SDHB subunit into the cytoplasm where it is rapidly degraded (13). The staining protocol used (XT UltraView DAB v3, Benchmark IHC/ISH module) included pre-treatment with cell conditioner 1, incubation with primary antibody at 37°C, followed by incubation with Prep Kit 517 solution for 32 min, followed by counterstaining with hematoxylin for 8 min.

Sanger sequencing for examination of LOH in tumors

DNA was extracted from samples using QIAamp DNA FFPE tissue kit (Qiagen). Using the AmpliTaq Gold 360 Master Mix (ThermoFisher Scientific), DNA was amplified by PCR targeting exon 5 of *SDHC* or exon 13 of *SDHA* (primers available upon request). After purification, the PCR products were sequenced using the Sanger method on a AB3500XL DX genetic analyser (ThermoFisher Scientific).

Comparison of patients with '3PAs' syndrome and 'non-3PAs' syndrome based on published data

The characteristics of patients who presented with PA and SDHx/MAX (likely) pathogenic variants were compared to patients with *AIP*, *MEN1*, *PRKAR1A*, *CDKN1B*-related PA and to patients with non-genetically determined PAs. The patients with non-genetically determined PAs came from the cohort reported by Daly *et al.* from a Belgian population (1). The *AIP* cases corresponded to 64 published cases with that phenotype (a list of references is available upon request). The *MEN1* cases were extracted from the UMD-MEN1 Database (14), the Carney complex cases were from a literature review published by Cuny *et al.* (15), and the MEN4 cases were from reviews conducted by Alrezk *et al.* and Fredericksen *et al.* (16, 17).

Statistical analysis

Statistical analyses were performed using Prism v6.0 (GraphPad Software). Patient characteristics were compared using the two-tailed Fisher's exact test for qualitative variables and the non-paired, non-parametric Mann-Whitney *U*-test for quantitative values.

Results

A total of 263 patients were included (Table 1 and Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). The mean age at diagnosis of PA was 29.3 years (min: 8; max: 78), and the mean age at genetic screening was 36.1 years (min: 8; max: 79). The occurrence of PA was sporadic in 227 patients (86.3%), while 36 patients presented with a familial history of PA (13.7%). By NGS sequencing, we found 295 single nucleotide variants, among which 7 variants were classified as pathogenic, 5 as likely pathogenic, and 7 as VUS (Fig. 1, Table 1 and Supplementary Tables 2, 3). We did not find CNV. Five PV and LPV were found in patients with a familial history of PA out of 36 and 7 in patients with sporadic PA out of 227. The probability to find a (likely) pathogenic variant was higher in cases of patients with a family history of PA compared to those with no history (odds ratio: 5.069, 95% CI: 1.69–15.79, $P=0.014$).

Among the sporadic cases, five mutations occurred in patients younger than 30 years (5/133) and two occurred in patients older than 30 years. Among the 12 PVs and LPVs, we found 7 variants in *AIP*, 2 in *MEN1*, 2 in *SDHA*, and 1 in *SDHC* (Supplementary Table 2). The medical

Clinical Study	G Mougél and others	<i>SDHx</i> genes in isolated pituitary adenoma	183:4	372
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Table 1 Clinical characteristics of the patients included in this study.

	Sporadic cases		Familial cases	Total
	<30 years	>30 years		
Number of patients (n)	133	94	36	263
Age at diagnosis (years) mean (range)	22.2 (9–30)	39.4 (31–78)	32.3 (8–77)	29.3 (8–78)
Sex ratio (male/female)	58 M/75 F (0.77)	62 M/32 F (1.9)	17 M/19 F (0.9)	135 M/128 F (1.05)
Size of pituitary adenoma				
Macro adenoma	95	70	22	187 (71.1%)
Micro adenoma	15	4	6	25 (9.5%)
NA	23	20	8	51 (19.4%)
Secretion of pituitary adenoma (n, %)				
PRL	52	20	14	86 (32.7%)
GH	31	36	5	72 (27.4%)
NFPA	8	6	8	22 (8.4%)
ACTH	21	3	2	26 (9.9%)
Mixed	4	8	1	13 (4.2%)
LH/FSH	1	4	0	5 (1.9%)
TSH	1	1	0	2 (0.8%)
NA	15	16	6	37 (14.1%)
Number of variants, all genes (n)	148	115	32	295
VUS	4	3	0	7
LPV	0	2	3	5
PV	5	0	2	7

CTH, adrenocorticotrophic hormone; F, female; FSH, follicular-stimulating hormone; GH, growth hormone; LH, luteinising hormone; LPV, likely pathogenic variant; M, male; NA, not available; NFPA, non-functional pituitary adenoma; PRL, prolactin; PV, pathologic variant; TSH, thyroid-stimulating hormone; VUS, variant of uncertain significance. Macroadenoma is defined by a diameter >10 mm; microadenoma is defined by a diameter <10 mm.

histories of the patients with three *SDHx* mutations are described subsequently and in Table 2.

Case presentation

Case 1

A 17-year-old previously healthy male patient presented with a recent history of severe right vision loss, clinically measured on the monoyer scale (right eye: 4/10, left eye: 10/10). MRI revealed a large (>10 mm) cystic pituitary mass, and his prolactin level was measured at 91 µg/L (reference range: 4.1–15.34 µg/L). The patient underwent transsphenoidal surgical debulking because of the visual defect. The post-operative examination was without complications and demonstrated a marked improvement in vision. The post-operative prolactin secretion was measured at 104 µg/L, probably explained by an incomplete resection. Post-operative MRI displayed post-operative reorganization without visible residue, resolved 6 months later. The histopathological examination of the resected tissue was consistent with a prolactinoma showing aggressive criteria (grade 1b under the new classification) (18). No neoplastic cells demonstrated vacuolated cytoplasm. The Ki-67 labeling index was 5%, and P53 was expressed in 10% of cells. Cabergoline was

initiated at a dose of 0.5 mg weekly, producing prolactin normalization (3 µg/L at 3 months).

A *SDHC*: c.405+1G>T, p.(?) heterozygous pathogenic variant was demonstrated. This variant was present in the population database at a weak allele frequency (Minor Allele Frequency – MAF <0.001%). This variant has been reported as causing the deletion of exon 5 and a shift in the reading frame (19). *SDHB* IHC analysis of the PA was positive (Fig. 1 and Table 2), while the search for LOH in the tumor was negative. A whole-body CT and investigation of serum and urinary catecholamines did not show signs of PPGL. The *SDHC* variant was present in the father, who is asymptomatic at the age of 46. Careful examination revealed no lesions, and plasma methoxyamines were normal. Imaging exploration has not to date been carried out.

Case 2

A 42-year-old male patient, with no personal or family history of endocrine disease, consulted due to an 18-month history of erectile dysfunction and decreased libido. The primary hormonal assessment found a low testosterone level (0.64 ng/mL) and a pituitary profile showing FSH of 1.3 IU/L, LH of 2.7 IU/L and prolactin of 84 µg/L, thus supporting a central origin. Cranial MRI showed a pituitary macroadenoma with a moderate

Figure 1

IHC of SDHB. (A) Pituitary adenoma of Case 1, (B) PA of Case 3, and (C) pheochromocytoma. In A and B, the SDHB IHC shows a strong granular cytoplasmic staining (arrow) in tumor cells suggesting the persistence of SDH complex expression in pituitary adenomas. In C, the SDHB IHC reveals a lack of staining in pheochromocytoma tumor cells and strong granular cytoplasmic staining in clusters of cortical adrenal cells entrapped in the tumor (arrow, positive control).

suprasellar extension but no visual pathway compression. In this clinical context, cabergoline at a dose of 0.5 mg weekly was initiated with a good medical and biological response. The analysis of *SDHA* revealed a heterozygous frameshift variant on exon 6 of the gene (c.757_758del, p.(Val253Cys*67)), resulting in a premature stop codon. This variant was absent from gnomAD and from the FREX database, thus not reported in the French population. It was therefore classified as likely pathogenic.

Case 3

This male patient was diagnosed with an isolated microprolactinoma at 37 years of age and had a notable

family history of PA, with macroprolactinoma in his family but without any history of PPGL. His brother died during surgery to remove a massive pituitary adenoma. Prolactin secretion was well-controlled under cabergoline (PRL < 10 µg/L), but due to side effects a change to treatment with bromocriptine was made. Subsequently, prolactin levels were poorly controlled due to poor adherence to therapy (16.6 to 40 µg/L). Neurosurgical removal was performed and histopathological analysis of the tumor did not show cytoplasmic vacuolization. Analysis of PPGL genes found a heterozygous missense variant in exon 13 of *SDHA* (c.1753C>T, p.(Arg585Trp)). This variant is present in the population database (gnomAD MAF: 0.0025%); however, *in silico* analysis predicted a deleterious impact and studies have reported this variant as pathogenic (20). Therefore, this variant was classified as likely pathogenic. SDHB IHC analysis was positive (Fig. 1 and Table 1), and the search for LOH by Sanger sequencing was negative. Family members were not available to allow a genetic family survey to be conducted.

Literature review and phenotypic features in '3PAs' syndrome

After reclassification of the variants using ACMG criteria (12) and excluding patients with VUS or (likely) benign variants, we found 23 published cases of '3PAs' syndrome with *SDHx* or *MAX* (likely) pathogenic variants (Supplementary Table 4) (5, 6, 7, 8, 9, 21, 22, 23, 24, 25, 26, 27, 28, 29). Among the 23 cases, 19 patients had *SDHx* (likely) pathogenic variants: 2 in *SDHA*, 9 in *SDHB* (39%, 9/23), 2 in *SDHC*, and 6 in *SDHD* (26%, 6/23); four patients had *MAX* pathogenic variants. PA occurred before PPGL in six cases (6/23, 26%). Moreover, we also found five published cases with an isolated PA and a mutation in *SDHx* (one in *SDHA* and four in *SDHB*); all of these had a family history of PPGLs (Supplementary Table 5) (6, 30, 31, 32).

Overall, the *SDHx/MAX* patients with PA included those harboring PPGL ($n=23$, Supplementary Table 4), those with a family history of PPGL ($n=5$, Supplementary Table 5), and those without PPGL context ($n=3$ from this study). Of these 31 *SDHx/MAX* patients, 6 had a family history of PA (Supplementary Table 4 and our case #3), and among these only one without PPGL history (our case #3). Cases included 16 females and 14 males (sex ratio: 1.1/1, the sex was not specified for one patient). Diagnosis of PA was on average at 42.4 years of age (range: 16(min) – 72 (max) years). There were 23 macropAs, representing 74% of cases and 4 microPAs (13%). The

Clinical Study	G Mougél and others	<i>SDHx</i> genes in isolated pituitary adenoma	183:4	374
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Table 2 Characteristics of patients harboring an isolated pituitary adenoma and *SDHx/MAX* likely pathogenic or pathogenic variants in this study: genetic exploration and functional analysis.

Characteristics	Case 1	Case 2	Case 3
Sex	M	M	M
Age at PA diagnosis	17	42	37
Size of PA	Macro	Macro	Micro
Secretion of PA	PRL	PRL	PRL
Familial PA	No	No	Father and brother: macroPRL nephew: microPRL
Familial PPGL	No	No	No
PRL at diagnosis (µg/L)	91	84	55
Medical treatment	Cabergoline (postoperative)	Carbergoline	Cabergoline, bromocriptine
Surgery	Yes	No	Yes
Results of <i>AIP</i> , <i>MEN1</i> , <i>CDKN1B</i> genetic testing	Normal	Normal	Normal
Gene	<i>SDHC</i>	<i>SDHA</i>	<i>SDHA</i>
Variant	c.405+1G>T, p.(?)	c.757_758del, p.(Val253Cys*67)	c.1753C > T, p.(Arg585Trp)
Classification*	PV	LPV	LPV
Histopathological examination	Yes	No	Yes
Hormonal status	PRL+		PRL+
ki67	5%		<1%
% of P53-positive cells	10%		<1%
IHC SDH	Positive	–	Positive
LOH	Negative	–	Negative

Htz, heterozygous; LOH, Loss of heterozygosity; LPV, likely pathogenic variant; M, Male; macro, macroadenoma, defined by a diameter >10 mm; micro, microadenoma, defined by a diameter <10 mm; PRL, prolactin; PV, pathogenic variant; SDHB IHC, SDHB immunohistochemistry. *Classification using ACMG guidelines for classification of sequence variants (12).

most frequent types were prolactinoma (19/31, 61%), GH-secreting adenoma and NFPA (5/31, 16% for both) (Supplementary Table 6).

Next, we compared these *SDHx/MAX* patients with those from published cases of genetically determined PAs (i.e. due to mutations in *AIP*, *MEN1*, *CDKN1B* or *PRKAR1A*) and non-genetically determined PAs. The age of occurrence of PAs in the *SDHx/MAX* patients (mean: 42.4 years, range: 16–72) was later than that for *AIP* patients (mean: 25.9 years, range: 10–60, $P<0.001$), or *MEN1* patients (mean: 34.2 years, range: 7–82, $P=0.024$), or *PRKAR1A* patients (mean 31 years, range: 16–55, $P=0.007$) (Fig. 2 and Supplementary Table 6).

The proportion of prolactinomas was identical in *SDHx/MAX* patients and in the control population from the cohort published by Daly *et al.* (66% vs 61%) (Supplementary Table 6) (1). However, the PAs of the 31 *SDHx/MAX* patients were larger (23/31 macroadenomas vs 29/68 in control population $P=0.002$). In fact, macroprolactinomas were more frequent in the *SDHx/MAX* population (15/31 vs 11/68 $P=0.0013$). The *SDHx/MAX* patients also presented a tendency to show an older age at PA diagnosis than in the control population (42.4 years vs 34.5 years) though this was not statistically significant ($P=0.08$).

Discussion

Though mutations in *MEN1*, *AIP*, *CDKN1B*, and *PRKAR1A* genes have been identified as causal factors in familial PA, in most cases, no genetic cause was identified. For example, *AIP* mutations were found in only 20% of FIPA cases (33). In France, according to the TENgen guidelines (Oncogenetic Network in Neuroendocrine Tumors), mutations in *AIP*, *MEN1*, and *CDKN1B* genes are investigated in patients with PA who have (1) familial presentation, (2) syndromic association, or (3) isolated and sporadic pituitary macroadenoma occurring before the age of 30. *PRKAR1A* is investigated only in those patients with a typical syndromic association. Of note, in our cohort, several patients aged older than 30 years, with sporadic and isolated PA, had genetic testing on the express request of the endocrinologist due to notably aggressive or drug-resistant PAs, which are features of *MEN1*- and *AIP*-related PAs. In our cohort, the prevalence of mutations in *AIP* and *MEN1* was consistent with the prevalence reported in the literature for patients with PAs and using similar inclusion criteria (*AIP*: 1.1–11.7%, *MEN1*: 0–3.4%) (34, 35, 36, 37, 38, 39).

Recently, a novel syndromic association termed ‘3PAs’ and involving PAs and PPGLs has been described in some

Figure 2

Comparison of age of occurrence of PAs in genetic syndromes and control patients. Control, patients with non-genetically determined PAs were selected as a reference from the patients described by Daly *et al.* from a Belgian population (1); AIP cases, 57 published cases (list available upon request); MEN1, MEN1 cases harboring pathogenic or likely pathogenic variants extracted from the UMD-MEN1 Database (14); PRKAR1A, Carney complex published cases listed by Cuny *et al.*; CDKN1B, 11 MEN4 cases listed in reviews by Alrezk *et al.* and Fredericksen *et al.* (16, 17). * $P < 0.05$, ** $P < 0.01$, **** $p < 0.0001$.

cases associated with germline mutations in SDHx/MAX genes (5, 6, 7, 9, 22). A literature review on patients with SDHx/MAX mutations showing '3PAs' syndrome found six patients in whom the PAs were present in patients with PPGLs, while five patients had an isolated PA (Supplementary Tables 4 and 5), suggesting a new gateway into SDHx/MAX-related diseases. These data raise several issues regarding: (1) the incidence of SDHx/MAX mutations in patients with isolated PAs, (2) the characteristics of patients with isolated PAs harboring these mutations and, consequently, (3) whether mutations in SDHx/MAX genes might be looked for in patients with isolated PAs.

Herein, we highlight for the first time the co-occurrence of SDHx germline mutations in patients with an isolated PA and without personal or familial history of PPGL. In our study, among 263 patients with isolated PAs, we found 3 (likely) pathogenic variants in SDHx genes: 2 in sporadic cases of PA and 1 in a patient with a strong family history of PAs. The prevalence rate of mutations was 1.1%. In 2015, Xekouki *et al.* studied the prevalence of SDHx germline mutations in a cohort of 168 patients with PA, including 143 patients with isolated and sporadic PAs, 3 patients with sporadic '3PAs' syndrome, and 22 patients with familial '3PAs' syndrome (5). Three SDHx mutations were identified in patients with familial '3PAs' syndrome.

No mutations were found in the patients presenting with isolated PAs, probably because the cohort included a high proportion of patients with ACTH-secreting PAs (118/168, 70%).

It should be noted that the presence of SDHx (likely) pathogenic variants in patients with PAs might also be due to a fortuitous association. In a study by Hoekstra *et al.*, nonsense SDHA mutations were found at a frequency of 0.5% in a healthy population (40). Moreover, potentially (likely) pathogenic variants in SDHx/MAX genes, such as loss of function variants, are registered in population databases including gnomAD. There may, therefore, be a risk of an incidental association between pituitary adenoma and the presence of SDHx germline mutations. However, we did not identify any SDHx/MAX (likely) pathogenic variants in a cohort of 239 patients who presented with hyperparathyroidism and who had no personal or family history of PPGL and PA (personal data). These data favor a non-random association between SDHx/MAX (likely) pathogenic variants and PAs.

It is also true that the involvement of SDHx/MAX genes in PA tumorigenesis remains unclear. We have shown here that the age of onset of PA in patients with SDHx/MAX mutations is higher than that for other tumour suppressor genes and is equivalent to that of controls. The loss of expression of SDH within tumor tissue was not established in all patients, as was the case for cases 1 and 3 in our study. In published cases, among the 24 patients with PA and germline mutations in SDHx, SDHB IHC and LOH testing in tumor samples were done for ten cases, but a loss of staining/LOH was not observed in two of these, which does not support the hypothesis of Knudson's double hit in these cases. Nevertheless, the *Sdhb*^{+/-} murine model is consistent with the involvement of SDHx mutation in pituitary tumorigenesis (5). At 12 months age, the *Sdhb*^{+/-} mice developed a hyperplastic adenohypophysis, as is classically found in AIP-deficient mice (41) and in human PAs due to AIP, PRKAR1A mutations or Xq26 microduplication (42, 43, 44). The adenohypophyseal cells of *Sdhb*^{+/-} mice showed several intranuclear abnormalities and strong cytoplasmic and nuclear HIF-1 α staining consistent with the activation of the pseudohypoxia pathway in SDHx-mutated tumors (5, 9, 45, 46, 47). Concerning MAX, there are no publications reporting PA associating germline MAX mutation and negative MAX IHC and/or LOH or second somatic MAX mutation in PA tissue. Nevertheless in humans, data on the increased risk of PA in patients with SDHx/MAX mutations is required to reach a conclusion on the need for pituitary gland monitoring in symptomatic and

asymptomatic carriers of *SDHx/MAX* mutations. Induced pluripotent stem cells (IPs) modified with the *SDHx/MAX* mutation and differentiated into pituitary cells may help to clarify the mechanisms of pituitary tumorigenesis in this syndrome.

On the other hand, *SDHx/MAX* genetic testing for patients with PA should be decided while also taking into account (1) the low penetrance of *SDHx*-related manifestations, (2) the possible anxiety generated by this information for the patient and their family, (3) the exposure to ionizing radiation related to lifetime monitoring, and (4) the cost of clinical follow-up.

Of the three variants identified in patients with isolated PA, two occurred in *SDHA* and one in *SDHC*. In the PPGL population, *SDHx* germline mutations accounted for approximately 15% of all cases and for half of the familial cases. *SDHB* and *SDHD* mutations have been reported to be the most common, *SDHA* and *SDHC* mutations being less frequent (48). On a large series of *SDHA*-PPGL patients, the penetrance was calculated at 10% at 70 years of age (49), while Benn *et al.* and Maniam *et al.*, using a Bayesian statistical approach, indicated an overall penetrance of 1.7% (95% CI: 0.8–3.8%) and 0.1–4.9%, respectively (50, 51). In the same study, Benn *et al.* calculated the *SDHC* penetrance at 8.3% (95% CI: 3.5–18.5%) (50). Consequently, the absence of PPGL in our patients with *SDHA* or *SDHC* mutations, as well as in their families, is not unexpected since the penetrance of *SDHA/SDHC*-related PPGL is low and the age of disease onset is late. Indeed the age at PPGL diagnosis in *SDHC*- and *SDHA*-related patients is usually 35–40 years. In our three patients, initial exploration did not reveal PPGL but a longer follow-up is required to conclude the absence of PPGL.

Therefore, until proven otherwise, these three patients are at risk of developing PPGL later and should benefit from long-term monitoring.

Indeed, data are lacking to determine if the increased risk of PPGL in families with *SDHx* mutations, without a family history of PPGL, is the same as that of a family with a history of PPGL, and if these patients require the same level of monitoring, especially for *SDHA* asymptomatic carriers. However, taking into account the current state of our knowledge, it seems obvious that the presence of *SDHx/MAX* (likely) pathogenic variants in patients with isolated PAs justifies screening for PPGLs via careful clinical examination, full-body imaging, and the measurement of urinary catecholamine levels (40). Additionally, the monitoring data of patients with *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* mutations, as secondary

findings in clinical exome and genome sequencing from the ACMG, should certainly provide some answers (52). In any case, patients with a PA must be carefully examined not only for their family history of PA, but also for a history of PPGL, particularly for those patients bearing macroprolactinomas. Taking into account the published data, in cases of a family history of PPGL, genetic screening for *SDHx/MAX* is an absolute requirement for a family member bearing an isolated PA. For those patients with an isolated PA and any family history of PPGL, the benefit of *SDHx/MAX* genetic testing remains to be assessed.

A rare condition that requires *SDHx/MAX* genetic testing in patients with PA appears to be the presence of intracytoplasmic vacuoles in tumoral cells, a particular histological phenotype that has been reported in *SDHx*-related PAs (7) (Supplementary Table 3). These vacuoles do not appear to be fragments of mitochondria or endoplasmic reticulum (6) and most likely represent autophagic bodies, due to pseudohypoxia (47, 53, 54). In our patients, these vacuoles were not observed, but taking into account the literature, as in renal carcinoma (48), vacuolization of the cytoplasm should lead to *SDHB* (+/–*SDHA*) IHC and *SDHx* genetic analysis being carried out.

In conclusion, we report for the first time *SDHx* mutations in patients bearing PA without any family or personal history of PPGL. The prevalence rate of 1.1% is similar to that of *MEN1* in this indication, raising the question of whether systematic genetic screening for *SDHx/MAX* is necessary for such patients. However, the question of incidental association remains unresolved. Data are lacking to determine the benefit of *SDHx/MAX* genetic testing of patients with isolated PA who have any family history of PPGL. Conversely, data on the increased risk of PA is needed to reach a conclusion on the pituitary gland monitoring in symptomatic and asymptomatic carriers of *SDHx/MAX* mutations. Meanwhile, we recommend a careful examination of patients with isolated PAs, not only on their family history of PAs but also on any history of PPGLs. Lastly, a family history of PPGL, as well as the presence of intracytoplasmic vacuoles in the PA, requires *SDHx/MAX* genetic testing for PA patients.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-20-0054>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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Clinical Study	G Mougél and others	SDHx genes in isolated pituitary adenoma	183:4	379
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