

Germline variants in familial pituitary tumour syndrome genes are common in young patients and families with additional endocrine tumours

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Abstract

Objective: Familial pituitary tumour syndromes (FPTS) account for 5% of pituitary adenomas. Multi-gene analysis via next-generation sequencing (NGS) may unveil greater prevalence and inform clinical care. We aimed to identify germline variants in selected patients with pituitary adenomas using a targeted NGS panel.

Design: We undertook a nationwide cross-sectional study of patients with pituitary adenomas with onset ≤ 40 years of age and/or other personal/family history of endocrine neoplasia. A custom NGS panel was performed on germline DNA to interrogate eight FPTS genes. Genome data were analysed via a custom bioinformatic pipeline, and validation was performed by Sanger sequencing. Multiplex ligation-dependent probe amplification (MLPA) was performed in cases with heightened suspicion for *MEN1*, *CDKN1B* and *AIP* mutations. The main outcomes were frequency and pathogenicity of rare variants in *AIP*, *CDKN1B*, *MEN1*, *PRKAR1A*, *SDHA*, *SDHB*, *SDHC* and *SDHD*.

Results: Forty-four patients with pituitary tumours, 14 of whom had a personal history of other endocrine tumours and/or a family history of pituitary or other endocrine tumours, were referred from endocrine tertiary-referral centres across Australia. Eleven patients (25%) had a rare variant across the eight FPTS genes tested: *AIP* (p.A299V, p.R106C, p.F269F, p.R304X, p.K156K, p.R271W), *MEN1* (p.R176Q), *SDHB* (p.A2V, p.S8S), *SDHC* (p.E110Q) and *SDHD* (p.G12S), with two patients harbouring dual variants. Variants were classified as pathogenic or of uncertain significance in 9/44 patients (20%). No deletions/duplications were identified in *MEN1*, *CDKN1B* or *AIP*.

Conclusions: A high yield of rare variants in genes implicated in FPTS can be found in selected patients using an NGS panel. It may also identify individuals harbouring more than one rare variant.

European Journal of
Endocrinology
(2017) 176, 635–644

Introduction

Clinically significant pituitary adenomas (PAs) affect 1 in 1000 individuals, with a mean age at diagnosis of 40 years (1). Historically, 5% of PAs have been recognised to occur within a familial pituitary tumour syndrome (FPTS) with germline mutations most commonly identified in *MEN1*, encoding menin and *AIP*, encoding aryl hydrocarbon receptor-interacting protein (2). However, the molecular genetic aetiology remains unknown in a large proportion of families.

Multiple endocrine neoplasia, type 1 (MEN1), is an autosomal dominant disorder with predisposition to parathyroid, pituitary and pancreatic tumours (3, 4). Less frequent MEN1 manifestations include other endocrine lesions such as adrenocortical tumours and non-endocrine lesions such as facial angiofibromas, collagenomas and lipomas. The disorder is due to germline heterozygous mutations in the *MEN1* gene (2). Germline *AIP* mutations account for 20% of familial isolated pituitary adenoma (FIPA) kindreds. Affected individuals typically have a young onset of growth hormone (GH)- or prolactin (PRL)-secreting tumours, and treatment resistance is common (5). *PRKARIA*, encoding the type 1A regulatory subunit of cAMP-dependent protein kinase A, is less commonly implicated in PA and loss-of-function mutations typically result in Carney's complex including lentiginos, myxomas and primary pigmented nodular adrenocortical disease (2, 6). Mutations in *CDKN1B*, encoding cyclin-dependent kinase inhibitor 1B (also known as p27^{Kip1}), are a rare cause of familial PA characterised by an MEN1-like phenotype labelled 'MEN4' (2, 7). The succinate dehydrogenase (SDH) genes recognised for their role in pheochromocytoma/paraganglioma (PHAEO/PGL) syndromes, gastrointestinal stromal tumours (GIST) and renal cell carcinoma (RCC) (8), have recently been implicated in pituitary tumorigenesis. Germline mutations in *SDHA*, *SDHB*, *SDHC* and *SDHD* (collectively, *SDHx*) have been found in both individuals and kindreds with combinations of PA and PHAEO/PGL, now termed the 3P association (3PAs) (9, 10, 11, 12).

Disease-causing genetic mutations have traditionally been identified through single- or staged-gene analysis via Sanger sequencing in patients suspected of FPTS. Phenotype has been used to select the most likely gene(s) for analysis. Single-gene testing is associated with low genetic yields of less than 5% in the sporadic PA setting (13, 14), whereas staged-gene testing may be time consuming and expensive.

Targeted next-generation sequencing (NGS) is rapidly supplanting Sanger sequencing. NGS offers a streamlined method capable of simultaneously interrogating multiple genes (15). We report the use of a dedicated NGS panel, comprising the major pituitary tumorigenesis genes, in a cohort of patients with suspected FPTS. The primary objective of the study was to describe the frequency and pathogenicity of rare variants in *AIP*, *CDKN1B*, *MEN1*, *PRKARIA* and *SDHx*.

Subjects and methods

Patients

Forty-four patients were referred by endocrinologists across Australia and investigated as part of an ongoing study approved by the St Vincent's Hospital Sydney Human Research Ethics Committee. Inclusion criteria were adults with PA and at least one of: (i) age of onset ≤ 40 years, (ii) other personal history of endocrine neoplasia or (iii) family history of endocrine neoplasia. Genetic counselling was provided prior to obtaining written consent. Treating clinicians provided clinical data, and patients were asked to complete a detailed family history questionnaire. Genetic testing was performed only on probands.

Next-generation sequencing panel

Using the Roche/NimbleGen SeqCap EZ Choice Library platform (<http://www.nimblegen.com/products/seqcap/ez/choice/index.html>), we developed a custom NGS panel incorporating eight FPTS genes (*AIP*, *CDKN1B*, *MEN1*, *PRKARIA*, *SDHA*, *SDHB*, *SDHC* and *SDHD*). Germline DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Midi Kit (Qiagen), and DNA libraries were prepared using the KAPA Library Preparation Kit (Roche). NGS was performed using Illumina's HiSeq 2500 platform, with pools of 12–24 samples per sequencing lane. Depth of coverage was >30 -fold in 97% of the targeted genomic region and >100 -fold in 91%.

Bioinformatic and rare variant analysis

Sequencing data were processed according to Genome Analysis Toolkit's (GATK) best practices, developed in-house on the DNAnexus (www.dnanexus.com) cloud-based analysis platform. Sequencing reads were aligned to the human genome (v hs37d5) via Burrows-Wheeler

Alignment (16) and Novosort, with GATK IndelRealigner and GATK BaseRecalibrator used to improve read alignment and quality. Single nucleotide variants and small insertions/deletions were identified and annotated with HaplotypeCaller v3.3 and Ensembl Variant Effector Predictor (VEP; v74) respectively. Data were filtered and prioritised using an in-house platform, *Seave* (www.seave.bio; Gayevskiy *et al.*, manuscript in preparation) according to <1% frequency in the Exome Aggregation Consortium (ExAC) database and *in silico* assessment of variant pathogenicity. To ascertain known clinical associations of variants, we consulted OMIM, ClinVar, Human Gene Mutation Database (HGMD), GeneReviews and published literature.

Clinical interpretation

Rare variants were classified into the following categories as per the Association for Clinical Genetics Science (ACGS) (17) with additional reference to the American College of Medical Genetics and Genomics (ACMG) guidelines (18): category (1) benign, (2) likely benign, (3) of uncertain significance, (4) likely pathogenic and (5) pathogenic. Rare variants were confirmed by Sanger sequencing. Subjects who wished to be informed of genetic results were notified by their treating endocrinologist. Referral to a dedicated cancer genetics services was recommended for patients with category 3–5 variants.

Multiplex ligation-dependent probe amplification (MLPA)

Patients with other personal/family history of endocrine neoplasia, or young-onset (<25 years) acromegaly, had further assessment of *AIP*, *MEN1* and *CDKN1B* by MLPA to exclude copy number variation (CNV), which might have been missed on NGS. Commercially obtained reagents and probe-mixes (MRC-Holland, The Netherlands) were used according to manufacturing instructions.

SDH and AIP immunohistochemistry

AIP or SDHA and SDHB immunohistochemistry was performed in patients with available tumour specimens and *AIP* or *SDHx* variants respectively. Commercially available mouse monoclonal antibodies against SDHB (ABCAM ab14714, clone 21A11, dilution 1 in 100) and SDHA (Mitosciences Abcam MS204, Clone 2E, dilution 1 in 1000) were used according to previously described

methods (19). Granular cytoplasmic staining was interpreted as positive (normal staining pattern), whereas absent staining in the presence of an internal positive control in non-neoplastic cells was interpreted as negative (indicating mitochondrial complex 2 dysfunction) (8). Negative SDHB staining represented loss of function of any of the four *SDHx* genes, whereas negative SDHA staining indicated *SDHA* loss-of-function mutations as observed elsewhere (8, 19, 20). For AIP, a mouse monoclonal antibody (Novus Biologicals NB100-127, clone 35-2, dilution 1 in 1500) was used. Cytoplasmic staining was classified using a previously described, semi-quantitative system (21, 22), according to intensity (negative, 0; weak, 1; moderate, 2 and strong, 3) and pattern (patchy, 1 and diffuse, 2). Low AIP immunostaining was defined by a total score ≤ 2 .

Results

Patient characteristics

The 44 patients comprised 25 women and 19 men, with a median age of 31 years (Table 1). Thirty patients were recruited based solely on PA onset before age 40 years. Of the remaining six patients under the age of 40 years, three also had a family history of pituitary tumour, two had a family history of other endocrine neoplasia and one had a personal history of primary hyperparathyroidism (PHPT). Of the eight patients older than 40 years, five had concomitant PHPT, two had a family history of PA and one had a personal history of PHPT and a family history of Cushing's disease. Patients with category 3–5 variants were more likely to have other personal/family history of endocrine neoplasia compared to patients without such variants. GH and PRL-secreting PAs predominated, particularly amongst those with category 3–5 variants. Of the 37 cases where PA size was known, 92% presented with macroadenomas (≥ 10 mm) and 46% with invasive tumours (Hardy's grade 3). There were three cases of atypical PAs, with two fulfilling all three World Health Organization criteria (Ki67 >3%, increased mitotic rate and positive p53 immunostaining) and one fulfilling two criteria (Ki67 and p53). Most patients required multiple treatment modalities.

Rare genetic variants

NGS detected 13 rare (population frequency <1%) variants in 11/44 patients (25%) in the FPTS

Table 1 Patient characteristics according to genetic status. Data are presented as *n* (%).

	Total, <i>n</i> =44	Patients with category 3–5 genetic variants, <i>n</i> =9	Patients without category 3–5 genetic variants, <i>n</i> =35
Median age, IQR (years)	31, 21–37	26, 21–34	32, 21–37
Number of females	25 (57)	6 (67)	19 (54)
Inclusion criteria			
Age <40 years	36 (82)	7 (78)	29 (83)
Age >40 years	8 (18)	2 (22)	6 (17)
PHx other endocrine neoplasia	7 (16)	2 (22)	5 (14)
FHx pituitary tumour	7 (16)	4 (44)	3 (9)
FHx other endocrine neoplasia	3 (7)	1 (11)	2 (6)
Tumour subtype			
GH secreting	19 (43)	5 (56)	14 (40)
PRL secreting	16 (36)	3 (33)	13 (37)
ACTH secreting	3 (7)	0 (0)	3 (9)
Non-functioning	5(11)	1 (11)	4 (11)
Functioning FSH secreting	1 (2)	0 (0)	1 (3)
Other tumour features			
Gigantism	4 (9)	3 (33)	1 (3)
GH/PRL co-secretion	4 (9)	2 (22)	2 (6)
Atypical by WHO criteria	3 (7)	2 (22)	1 (3)
Hardy's grade			
I (microadenoma)	8 (18)	2 (22)*	6 (17)
II (macroadenoma)	17 (39)	5 (56)	12 (34)
III (sphenoid or cavernous invasion)	17 (39)	1 (11)	16 (46)
IV (metastatic)	0 (0)	0 (0)	0 (0)
Unknown	2 (5)	1 (11)	1 (3)
Treatment			
Monitoring	2 (5)	2 (22)	0 (0)
Medical	23 (52)	5 (56)	28 (80)
Surgical	18 (41)	5 (56)	23 (66)
Radiotherapy	11 (25)	2 (22)	14 (40)
>1 operation	9 (21)	2 (22)	7 (20)
>1 radiotherapy course	1 (2)	0 (0)	1 (3)

*Both patients diagnosed over age 30 and had a family history of pituitary tumour.

ACTH, adrenocorticotrophin hormone; FHx, family history; GH, growth hormone; IQR, interquartile range; PHx, past history; PRL, prolactin; WHO, World Health Organization.

genes: *AIP* (p.A299V, p.R106C, p.F269F, p.R304X, p.K156K, p.R271W), *MEN1* (p.R176Q), *SDHB* (p.A2V, p.S8S), *SDHC* (p.E110Q) and *SDHD* (p.G12S) (Table 2). This included two patients who each had dual variants (*AIP* p.R106C/*MEN1* p.R176Q and *AIP* p.A299V/*SDHD* p.G12S), and two variants which were each found in two patients (*AIP* p.R304X and *SDHC* p.E110Q). Three rare variants (all *AIP*) were classified pathogenic (category 5). Four were classified as variants of uncertain significance (VUS, category 3), including two novel *AIP* variants (p.R106C and p.K156K), an *SDHB* variant (p.A2V) and an *SDHC* variant (p.E110Q). The four remaining variants were classified as likely benign (category 2). Overall, 9 of 44 patients (20%) had potentially clinically relevant variants (category 3–5), which will lead to referral to a genetics service and potentially family segregation studies or definitive predictive genetic testing.

The six *AIP* variants included a known truncating mutation (p.R304X) in a mutational hotspot detected

in two patients with gigantism. The other pathogenic variants were a synonymous variant (p.F269F) predicted to result in aberrant splicing, found in a woman with gigantism and a 7 cm PA at age 15 years and a previously published missense variant (p.R271W) in a 21-year-old man with acromegaly. Two novel *AIP* variants were considered VUS: a missense variant (p.R106C, Supplementary Fig. 1, see section on supplementary data given at the end of this article) in a man with an atypical macroprolactinoma at 31 years and a synonymous variant (p.K156K) in a 26-year-old woman with a non-functioning PA and family history of PA. Although not previously described, p.R106C is exceedingly rare and multiple *in silico* prediction models support this variant as pathogenic. Protein structure analysis of the wild-type and mutant amino acids demonstrates differences in size, charge and hydrophobicity that are predicted to cause loss of interactions (Supplementary Fig. 2).

Table 2 Clinical features of patients with rare genetic variants in the pituitary tumorigenesis genes and evidence of pathogenicity for each variant.

Clinical features ^a	Gene (transcript id)	Genomic location (dbSNP id)	Variant position	In silico prediction ^b	ExAC frequency	PROVEAN score	CADD score	Evidence in literature	Clinical category ^c
21 years female with NF PA. No FHx endocrine neoplasia.	<i>AIP</i> (NM_003977.2)	g.67258367C>T (rs148986773)	c.896C>T; p.A299V	Tolerated ^d / benign [*]	4.28 x 10 ⁻⁴	-2.32	17.37	Mixed	2
31 years male with PRL PA. Father with PHPT.	<i>SDHD</i> (NM_003002.3) <i>AIP</i> (NM_003977.2)	g.111957665G>A (rs34677591) g.67256774C>T (rs369414668)	c.34G>A; p.G12S c.316C>T; p.R106C	Tolerated ^d / benign [*] Deleterious ^d / probably damaging [*]	0.7 x 10 ⁻² 3.52 x 10 ⁻⁵	0.21 -7.34	0.99 22	Benign None (novel)	2 3
13 years female with GH PA. No FHx endocrine neoplasia.	<i>MEN1</i> (NM_000244.3) <i>AIP</i> (NM_003977.2)	g.64575505C>T (rs607969) g.67258381C>T (rs104894195)	c.527C>T; p.R176Q c.910C>T; p.R304X	Tolerated ^d / benign [*] Truncating	1.2 x 10 ⁻² 1.72 x 10 ⁻⁵	-0.75 N/A	22.2 20.6	Mixed Pathogenic	2 5
25 years male with GH PA. No FHx endocrine neoplasia.	<i>AIP</i> (NM_003977.2)	g.67258278C>T (rs139407567)	c.807C>T; p.F296F	Splice site [†]	5.93 x 10 ⁻⁴	N/A	22.3	Pathogenic	5
26 years female with NF PA. Cousin with PRL PA.	<i>AIP</i> (NM_003977.2)	g.67256926G>A	c.468G>A; p.K156K	Splice site [†]	Not reported	N/A	11.54	None (novel)	3
21 years male with GH PA. No FHx endocrine neoplasia.	<i>AIP</i> (NM_003977.2)	g.67258282C>T (rs267606579)	c.811C>T; p.R271W	Deleterious ^d / possibly damaging [*]	Not reported	-7.38	20.2	Pathogenic	5
49 years female with PRL PA and PHPT. Aunt with ACTH PA.	<i>SDHB</i> (NM_003000.2)	g.17380510G>A (rs199948437)	c.5C>T; p.A2V	Deleterious ^d / benign [*]	2.07 x 10 ⁻⁵	-0.8	17.64	None	3
70 years male with PRL PA. 36 years daughter with PRL PA.	<i>SDHB</i> (NM_003000.2)	g.17380491G>A (rs148738139)	c.24C>T; p.S8S	Splice site [#]	0.4 x 10 ⁻²	N/A	0.42	Benign	2
34 years female with PRL PA. Brother with PRL PA.	<i>SDHC</i> (NM_001278172)	g.161332307G>C (rs201210474)	c.403G>C; p.E110Q	Deleterious ^d / possibly damaging [*]	0.5 x 10 ⁻²	-0.04	2.17	Uncertain	3
63 years female with GH PA, pituitary gangliocytoma, and PHPT. No FHx endocrine neoplasia.									

^aPA, pituitary adenoma, type indicated by hormone secretion; NF, non-functioning; PRL, prolactin; GH, growth hormone; ACTH, adrenocorticotropic hormone. ^bModels of prediction: ^dSIFT, ^{*}PolyPhen, [†]ALAMUT and [#]MutationTaster. ^cClinical category as per ACMG classification: 5, pathogenic; 4, likely pathogenic; 3, uncertain significance; 2, likely benign; 1, benign. PHPT, primary hyperparathyroidism.

The p.K156K variant is not reported in the ExAC database and is predicted to affect splicing by Alamut. The remaining *AIP* missense variant (p.A299V) in a 21-year-old woman with a non-functioning PA was classified as likely benign. This patient also carried an *SDHD* variant (p.G12S), classified likely benign based on its frequency in healthy controls and *in silico* prediction models, although there is some evidence for tumorigenesis beyond the pituitary (23). *AIP* immunohistochemistry showed low expression in the PAs from the patients with the p.R106C (classified as VUS) and p.A299V (category 2) variants (Supplementary Fig. 3) and moderate expression in the PA corresponding to the p.R304X (category 5) variant.

The *MEN1* variant (p.R176Q) was classified as a likely benign variant and was found in the patient who also harboured the novel *AIP* p.R106C VUS. His father had PHPT, which raised the initial suspicion of *MEN1* in this kindred, as well as a history of papillary thyroid cancer.

Two variants were identified in *SDHB*. The missense variant (p.A2V) occurred in a 49-year-old female with a microprolactinoma, PHPT and a family history of Cushing's disease. It was classified as a VUS because of its rare frequency and *in silico* models predicting possibly damaging protein alterations. The synonymous *SDHB* variant (p.S8S) in a 70-year-old man with a personal and family history of prolactinoma was classified as likely benign based on evidence from ClinVar and its frequency in control populations (24), although possible aberrant splicing is predicted by MutationTaster. A missense *SDHC* variant (p.E110Q) was found in two patients: a 34-year-old female with a personal and family history of prolactinoma and a 63-year-old female with acromegaly associated with a pituitary gangliocytoma and previous PHPT. *In silico* prediction favoured a deleterious or possibly damaging alteration by the SIFT and PolyPhen tools, respectively, whereas Mutation Taster considered this variant to be a polymorphism. In the absence of further evidence regarding this variant, it was classified as a VUS. None of the patients with an *SDHx* rare variant had a personal/family history of PHAEO/PGL. *SDHA* and *SDHB* immunohistochemistry was performed on parathyroid tumour tissue from the 49-year-old woman with the *SDHB* p.A2V VUS and both pituitary and parathyroid tumour tissue from the 63-year-old woman with the *SDHC* p.E110Q VUS. All three tumour specimens demonstrated preserved staining, and none contained the intracytoplasmic vacuoles previously reported in *SDHx*-mutated PAs (9).

MLPA of *AIP*, *CDKN1B* and *MEN1* was performed in 15 patients with no deletions or duplications identified.

Discussion

We describe the use of a custom NGS gene panel in a selected cohort of patients with PA. Our results demonstrate a high frequency (20%) of patients with rare germline variants of potential clinical relevance (category 3–5) in eight pituitary tumorigenesis genes. Of note, these variants were not distributed across the eight genes tested, but they were instead limited to three genes – *AIP*, *SDHB* and *SDHC* – with an additional category 2 variant found in *MEN1*. The high genetic yield in this study suggests that germline variants in FPTS genes may play a more significant role in PA predisposition than previously thought. To our knowledge, only one other study has employed an NGS gene panel for patients with suspected FPTS and no mutations were found, despite the inclusion of 14 genes (including those analysed in the present study) and recruitment of only patients with personal and family histories of PA, although the median age was older at 55.6 years (25). The discrepancy between the studies may also relate to sequencing quality and bioinformatic analysis of the raw genome data. All variants in our study were validated by Sanger sequencing, demonstrating that our approach has high diagnostic specificity.

Genetic testing has been recommended in patients with young-onset PAs for several years now (26, 27). Our findings of known pathogenic *AIP* mutations in four patients aged 13–25 years, all with apparently sporadic GH tumours, support recommendations that genetic testing in sporadic PA patients be restricted to those below age 30 years with macroadenomas (5, 28). We originally chose to include sporadic cases under age 40 years to satisfy current age-based recommendations for genetic testing and because little is known about the age penetrance of pituitary tumours in the context of *SDHx* germline mutations. Our concern was that genetic testing recommendations may be biased towards detecting *AIP* kindreds. In a recent large series, Xekouki *et al.* reported three patients with familial 3PAs associated with *SDHx* mutations, two of whom developed their first tumours after age 35 years (12). We also found two rare variants, albeit of uncertain significance, in *SDHB* (p.A2V) and *SDHC* (p.E110Q) in three patients, aged 34–63 years. Amongst the nine patients with rare category 3–5 variants, there were just two microadenomas: both in patients with the *SDHC* variant (p.E110Q) and a family history of pituitary tumour. In fact, of the five patients with rare variants (all category 2–3) who were aged 30–40 years, all had other personal/family history of endocrine neoplasia, which may serve as age-independent indications for genetic testing.

In our cohort, rare variants in *AIP* were most common with six in total. This included a known pathogenic truncating variant (p.R304X) resulting in a very short half-life, decreased anti-proliferative activity and loss of the AIP–PDE4A5 interaction in the mutant protein (22, 29). This mutation has been implicated in numerous sporadic and familial cases of PA, particularly in the setting of gigantism as observed in both of our cases (5, 13, 22, 30). The pathogenic missense variant in *AIP* (p.R271W) has been similarly described in multiple FIPA families (5, 30) and results in a significantly shortened protein half-life (29). The pathogenic synonymous variant (p.F269F) is predicted to affect splicing and loss of exon 6 with reduced protein expression (22). It has been described in FIPA kindreds and sporadic PA cases (5, 22, 28), including the setting of gigantism as in our case (14). Another synonymous variant (p.K156K) was also predicted to interfere with splicing; however, it was classed as a VUS due to insufficient supporting evidence as it is novel. An additional novel *AIP* variant (p.R106C), also classed as a VUS, was predicted to be deleterious on *in silico* analysis and maps to an Hsp90-binding site (Supplementary Fig. 1), which is thought to be critical to the tumour suppressor function of AIP (31). Another missense *AIP* variant (p.A299V) was classified as likely benign as there is some evidence for a reduced half-life of the mutant protein (29), but its ExAC frequency of 1/2500 is higher than expected for disease frequency and *in silico* analysis predicted it to be benign.

AIP expression was present but reduced in all three PAs (associated with category 2, 3 and 5 variants) in which AIP immunohistochemistry was performed. Although absent protein expression supports loss-of-function variants, even truncating variants such as p.R304X have been described to result in some persistent AIP expression (21, 22), as was found in our patient with the same variant. The dichotomy of *AIP* genetic variants and intact protein expression may relate to the position of the immunohistochemistry epitope target relative to the variant position (21). There may also be treatment effects as somatostatin analogue use has been associated with increased AIP expression *in vivo* and *in vitro* (32). Conversely, loss of AIP expression may be found in the absence of germline *AIP* variants, particularly in aggressive sporadic GH-secreting PAs and sporadic prolactinomas (21). Our findings further support the evidence that the sensitivity and specificity of immunohistochemistry are insufficient to assess *AIP* variant pathogenicity.

Our finding of two category 3 *SDHx* variants (p.A2V in *SDHB* in one patient and p.E110Q in *SDHC* in two

patients) was not expected: the patients had personal/family histories of other endocrine tumours, but this did not include PHAEO/PGL. Dénes *et al.* found germline *SDHx* variants in 41% of patients with sporadic or familial PA and PHAEO/PGL (9). Xekouki *et al.* (12) found *SDHx* germline variants in familial, but not sporadic, cases of 3PAs. Intriguingly, the remaining 143 patients with sporadic PA in the absence of PHAEO/PGL collectively demonstrated 22 polymorphisms in *SDHB*, 16 in *SDHC* and 7 in *SDHD*. We also found an additional two likely benign *SDHx* variants in our PA patients. Unlike PHAEO/PGL and GIST, where SDH deficiency on immunohistochemistry occurs in 5–15% of cases, SDH deficiency is rare in PA and RCC where it occurs in <1% of unselected cases (8, 19, 20). Nonetheless, *Sdhb*+/- mice develop pituitary hyperplasia (12), though it is unknown if this translates into a hyperplasia–adenoma sequence as described in other aetiologies (33). These observations together with our findings suggest that *SDHx* variants may be contributory, but not exclusively causative, in pituitary tumorigenesis. Analogous to AIP immunohistochemistry, retained SDH tumour expression does not exclude pathogenic *SDHx* variants.

The pathogenicity of the *MEN1* missense variant (p.R176Q) is debated. *In silico* tools including PolyPhen, SIFT and PROVEAN all predicted it to be benign, and its frequency is similar between clinical *MEN1* and control populations (34). However, p.R176Q has been implicated in patients with *MEN1*-related tumours in the absence of other *MEN1* variants (35), but with no functional differences between the mutant and wild-type proteins (36), leading to it sometimes being considered a pathogenic variant of low penetrance. Polymorphisms in *MEN1*, and other genes such as *DRD2* and *SSTR5*, have been associated with increased risks of developing PAs (37). In our study, the *MEN1* p.R176Q variant was found in the same patient carrying the p.R106C *AIP* VUS. Although the patient's father had multi-gland PHPT, the proband had an atypical macroprolactinoma. We considered the possibility of mutational load in the proband, where the *AIP* and *MEN1* variants may have acted synergistically to produce the more severe phenotype of a highly aggressive PA. This finding of dual *AIP* and *MEN1* variants would have been masked if single-gene analysis was undertaken, where *MEN1* would have been selected based on family history.

Multi-gene analysis through NGS facilitates the detection of variants not informed by phenotype. We found another young patient with dual rare variants in *AIP* (p.A299V) and *SDHD* (p.G12S), although both were of

category 2 pathogenicity. Additionally, we identified an *SDHB* rare variant (p.A2V, classified as VUS) in a 49-year-old woman with an *MEN1* phenotype, leading to further family segregation studies, whereas there would have been no indication for *SDHx* screening in traditional clinical practice. Even if staged Sanger sequencing was pursued, the monetary and time costs would have increased considerably compared to NGS. The efficiency of NGS beyond Sanger sequencing will accelerate as the number of genes implicated in FPTS grows.

Other studies have demonstrated that even large genetic deletions can be detected using NGS (38), and the overall depth of coverage of our NGS panel was exceptional; however, MLPA may be considered in highly suspicious cases with uninformative NGS results. MLPA is recommended in all individuals with clinical *MEN1* as up to 4% of patients with a clinical diagnosis of *MEN1* have large genetic deletions that are undetectable on Sanger sequencing (39). Large or whole-gene deletions have also been described in *AIP* (5). We performed MLPA in a subset of patients more suspicious for clinical *MEN1* and FIPA syndromes but no CNV was detected in *MEN1*, *CDKN1B* or *AIP*. We did not extend MLPA analysis to specifically screen patients for other potentially relevant CNVs such as *PRKAR1A* and *SDHx* deletions (40, 41), or Xq26.3 microduplications which account for 10% of gigantism (42). None of our patients had phenotypes suggestive of Carney's complex or 3PAs, which are associated with *PRKAR1A* and *SDHx* mutations, respectively. Moreover, infantile-onset gigantism, associated with Xq26.3 microduplications, was absent in our cohort (42). Although our NGS panel may have failed to capture such larger CNVs, we have been optimising algorithms to reliably detect variable-length CNVs using the panel data, which should obviate the need for additional MLPA analysis in the future (43, 44). NGS of germline DNA may also miss somatic aberrations such as *GNAS* mosaicism; however, no patients demonstrated features of McCune–Albright syndrome to suggest this.

This study had a relatively small sample size. Nonetheless, recruited patients reflected the population we wished to target for genetic testing. In clinical practice, young patients with sporadic PA predominate over those with a personal history of other endocrine tumours and/or a family history of pituitary or other endocrine tumours. Genetic testing is often considered in patients with a family history of PA, as in six (13.6%) of our cohort. Yet, over half of our patients with rare variants had no family history of PA. A family history of non-endocrine tumours may also be relevant in regards to genetic syndromes such

as Carney's complex. As this was not part of the inclusion criteria for the present study, such cases may not have been captured. Finally, we acknowledge selection bias may be present with enrichment of cases considered by their treating clinicians to have a higher likelihood of carrying a germline mutation. This is particularly as genetic testing in patients with pituitary tumours is not routinely considered unless features of *MEN1* are also present.

As the genetics underpinning pituitary tumorigenesis is a developing area, use of the ACMG guidelines to assess variant pathogenicity was challenging. Many of the criteria cannot be assessed due to the lack of a body of literature, such as the presence of definitive evidence from *in vitro* or *in vivo* functional studies (18). As family members were not tested as part of this study, we also lacked segregation data. This resulted in significant reliance on *in silico* models; however, this form of evidence is weighted much less by the ACMG guidelines.

We propose that use of an NGS gene panel should be standard care (supplemented by variant validation using Sanger sequencing) in patients under the age of 30 years with sporadic PA or those, of any age, with a personal history of other endocrine tumours and/or a family history of pituitary or other endocrine tumours. The successful uptake of NGS testing will require access to experienced clinical bioinformaticians and molecular pathology expertise to appropriately evaluate variant pathogenicity, ideally via a multidisciplinary setting.

Future directions of research include evaluation of the role of mutational load and gene–gene interactions as they pertain to pituitary tumorigenesis. Familial segregation studies will be undertaken to clarify VUS found in the present study. Furthermore, our NGS panel will continue to evolve to study the contribution of new genes, such as *GPR101*, which are implicated in pituitary tumorigenesis. As we continue to utilise our NGS panel in patients with PAs, it will be of interest to see if the high genetic yield in this initial study is maintained across a larger cohort.

Given germline variants in pituitary tumorigenesis genes are associated with earlier onset, more aggressive disease and potentially other endocrine tumours, the genetic information revealed by NGS will have important prognostic implications for the individual. Subsequent predictive genetic testing in families may also lead to earlier diagnosis and the potential for treatment at a time when cure may be feasible. On a broader level, the capacity of NGS to assess multiple genes simultaneously may improve our understanding of pituitary tumorigenesis, which may lead to new therapeutic avenues for patients with PA.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-16-0944>.

Declaration of interest

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

Funding

This work was partially supported by the Cancer Institute NSW through the Sydney Vital Translational Cancer Research Centre; a project grant through Cancer Council NSW and an infrastructure grant through Therapeutics Innovation Australia. The Kinghorn Centre for Clinical Genomics is supported by the Kinghorn Foundation. S M C D is supported by a National Health and Medical Research Council scholarship. M J C is supported by a fellowship from the Cancer Institute of NSW.

Acknowledgements

The authors thank Queensland Pathology, particularly Jody Sawyer, for Sanger sequencing for validation of *MEN1* variants as well as referring clinicians, including Carmela Caputo, Daniel Chen, Warrick Inder, Kylie McLachlan, Felicity Pyrlis, Robert Schmidli, Lyndal Tacon and Venessa Tsang. They also thank Jiang Tao, Kerith-Rae Dias, Paula Morris and Kevin Ying of the Kinghorn Centre for Clinical Genomics at the Garvan Institute of Medical Research for their invaluable expertise, assistance and training in NGS library preparation and bioinformatics.

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Received 18 November 2016

Revised version received 8 January 2017

Accepted 17 February 2017