Germline Variants in Sporadic Pituitary Adenomas

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Abstract

Context: Data on germline genetics of pituitary adenomas (PAs) using whole-exome sequencing (WES) are limited.

Objective: This study investigated the germline genetic variants in patients with PAs using WES.

Methods: We studied 134 consecutive functioning (80.6%) and nonfunctioning (19.4%) PAs in 61 female (45.5%) and 73 male patients (54.5%). Their median age was 34 years (range, 11-85 years) and 31 patients had microadenomas (23.0%) and 103 macroadenomas (77%). None of these patients had family history of PA or a known PA-associated syndrome. Peripheral blood DNA was isolated and whole-exome sequenced. We used American College of Medical Genetics and Genomics (ACMG) criteria and a number of in silico analysis tools to characterize genetic variant pathogenicity levels and focused on previously reported PA-associated genes.

Results: We identified 35 variants of unknown significance (VUS) in 17 PA-associated genes occurring in 40 patients (29.8%). Although designated VUS by the strict ACGM criteria, they are predicted to be pathogenic by in silico analyses and their extremely low frequencies in 1000 genome, gnomAD, and the Saudi Genome Project databases. Further analysis of these variants by the Alpha Missense analysis tool yielded 8 likely pathogenic variants in 9 patients in the following genes: *AIP*:c.767C>T (p.S256F), *CDH23*:c.906G>C (p.E302D), *CDH23*: c.1096G>A (p.A366T), *DICER1*:c.620C>T (p.A207V), *MLH1*:c.955G>A (p.E319K), *MSH2*:c.148G>A (p.A50T), *SDHA*:c.869T>C (p.L290P) and *USP48* (2 patients): c.2233G>A (p.V745M).

Conclusion: This study suggests that about 6.7% of patients with apparently sporadic PAs carry likely pathogenic variants in PA-associated genes. These findings need further studies to confirm them.

Key Words: pituitary adenoma, germline variants, pituitary gland tumors, genetics, molecular genetics of pituitary adenomas

Abbreviations: ACMG, American College of Medical Genetics and Genomics; ACRO, acromegaly; ACRO-PL, acromegaly-prolactinoma (somatolactotroph adenoma); ACTH, adrenocorticotropin; CD, Cushing disease; FIPA, familial isolated pituitary adenoma; FSH, follicle-stimulating hormone; GH, growth hormone; GN, gonadotropinoma; LH, luteinizing hormone; LS, Lynch syndrome; NGS, next-generation sequencing; PA, pituitary adenoma; PL, prolactinoma; PPGL, paragangliomas and pheochromocytoma; SGP, Saudi Genome Project; TSH, thyrotropin; TSHoma, TSH-secreting adenoma; VUS, variant of unknown significance; WES, whole-exome sequencing.

Pituitary adenomas (PAs) are common, representing about 10% to 16% of intracranial tumors [1, 2]. Considered to be primarily sporadic tumors for a long time, it has become clear over the last decade that a significant proportion of PA carry germline (~5%), mosaic (<1%) or somatic genetic alterations (~40%) [3]. Germline mutations occur in familial syndromes that are known to be associated with PA (eg, *MEN1*) or in nonsyndromic cases with isolated PAs [3, 4]. This latter group may occur in families without known genetic syndrome and is

commonly referred to as familial isolated PA (FIPA) [3, 5]. FIPA refers to PA when 2 or more members of the family are affected by PA without other manifestations of a known familial syndrome [5]. It also includes apparently sporadic cases (also called simplex cases) of patients with no, unknown or unrecognized family history of PA but with underlying germline mutations in PA-predisposing genes [2, 3, 6]. FIPA is described as homogenous when PA in different affected members are functionally the same (eg, prolactinoma) and

heterogeneous when PA is of different secretory subtypes [5, 7]. Overall, about 15% to 25% of nonsyndromic FIPA occur in patients with germline mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene [6, 8, 9] or in patients with X-linked acrogigantism (XLAG) due to microduplication of *GPR101* [10]. About 50% of homogenous somatotropinoma FIPA kindreds have *AIP* mutations or *XLAG* duplications [8, 11]. *AIP* mutations have low penetrance (~20%) [6, 11], and this has 2 implications. First, cases with *AIP* mutations may present as sporadic PA since other family members who carry *AIP* mutations may not manifest the disease. Second, isolated cases with underlying germline *AIP* mutations may be considered sporadic PAs if not tested for *AIP* mutations.

Interestingly, *AIP* mutations are extremely rare as truly de novo [12]. Most cases with FIPA and sporadic PA have no identifiable germline genetic alterations [3]. Although there are many studies that have assessed the underlying genetic aberrations of PAs focusing on a limited number of genes, studies of the germline genetics of PAs using the more comprehensive next-generation sequencing (NGS) are limited. For this reason, we undertook this study to systematically assess the prevalence of known PA-associated germline mutations and to look for new genetic alterations in a large series of patients with PAs.

Materials and Methods

Patients

After obtaining an ethical approval from the Office of Research Affairs of the King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia (ORA-2130012), and patients signed written informed consents, we collected 5 mL blood in EDTA-impregnated tubes for DNA isolation from 134 consecutive patients. To decrease heterogeneity of the study sample and since our aim was to investigate molecular genetics of sporadic PAs, we excluded patients with known syndromes and patients with a family history of PA or other tumors that might be part of a syndrome involving the pituitary gland including patients with primary hyperparathyroidism; neuroendocrine tumors of the lungs, thymus, pancreas, or gastrointestinal tract; pheochromocytoma or paragangliomas; neurofibromatosis; pulmonary blastomas; or skin changes suggestive of Carney complex or neurofibromatosis.

The histopathological examination included the standard hematoxylin and eosin staining and immunohistochemistry for different anterior pituitary hormones (adrenocorticotropin [ACTH], thyrotropin [TSH], luteinizing hormone [LH], follicle-stimulating hormone [FSH], growth hormone [GH], and prolactin), cytokeratin (CAM5.2), and Ki67. Staining for pituitary transcription factors was not consistently performed. The diagnosis of different types of PA was based on plasma levels of pituitary hormones and immunohistochemistry of the resected PA when available. In case of normal plasma pituitary hormones, the diagnosis was based on immunohistochemical staining. Somatotroph adenomas are those that stain for GH and are negative for other pituitary hormones. Lactotroph adenomas stain for prolactin only. Somatolactotroph adenomas stain for GH and prolactin. Patients with elevated GH, insulin-growth factor-1 (IGF-1), and prolactin of less than 100 ng/dL but with negative staining for prolactin on immunohistochemistry were diagnosed as somatotroph adenomas only (not somatolactotroph adenomas), and the prolactin elevation was presumed to be due to pituitary stalk compression. Gonadotroph adenomas were diagnosed in patients with no clinical features of hormonal hypersecretion with or without elevated plasma levels of LH and FSH and immunohistochemical examination showing positive staining for gonadotropins with negative staining for other hormones. Nonfunctioning PAs were diagnosed in patients without clinical features of hormonal hypersecretion, normal plasma levels of pituitary hormones, and negative immunohistochemistry for all pituitary hormones. Corticotroph PA stains for ACTH only in a patient with or without Cushing syndrome, and TSH-secreting adenomas (TSHoma) stains for TSH only in a patient with or without evidence of clinical and biochemical hyperthyroidism with nonsuppressible TSH.

DNA Isolation

Genomic DNA was extracted from peripheral blood samples using a commercial DNA extraction kit (QIAamp Blood Midi Kit, Qiagen) according to the manufacturer's instructions. The extracted DNA was evaluated for quality (A_{260}/A_{280} ratio ≥ 1.8) and quantified using a nanodrop-1000 spectrophotometer.

Whole-Exome Sequencing

In 14 patients, we used ready variant calling WES data from previous work using the Ion Torrent platform (AmpliSeq kit). The methods were as previously described [13]. Briefly, 100 ng DNA were amplified using AmpliSeq HiFi mix (Life Technologies) for 10 cycles. The polymerase chain reaction products were pooled, followed by primer digestion using FuPa reagent (Life Technologies). This was followed by a ligation step using Ion P1 and Ion Xpress Barcode adapters. The library was purified and quantified using quantitative polymerase chain reaction and the Ion Library Quantification Kit (Life Technologies). The emulsion of the libraries was performed by the Ion OneTouch System to attach the DNA fragments to the Ion Sphere particles. The final step in the library preparation was the enrichment of the Ion Sphere particles using Ion OneTouch ES (Life Technologies). Following that, the library was loaded on the sequencing chip, which was then inserted into the Ion Proton instrument (Life Technologies) for sequencing.

For the remaining 120 of 134 patient samples, we used an Illumina NovaSeq 6000 platform as follows: DNA pair-end fragment libraries were prepared according to Illumina WES protocol using the TruSeq Exome Kit (catalog No. 20020615, Illumina Inc) with a target insert size of 150 bp. DNA sequencing was performed using the Illumina NovaSeq 6000 platform (S2 reagent kit, 200 cycles configuration, and target sequencing depth of 50×). The details of DNA sequence quality assessment and bioinformatics analysis are included in supplementary file 1 [14].

Statistical Analysis

Continuous variables are expressed in median and ranges. Categorical values are expressed in numbers and percentages. We used Statistical Package for Social Sciences (SPSS version 20, IBM) to analyze the data. A 2-tailed *P* value of less than .05 was considered statistically significant.

Results

Patients

We enrolled 134 patients with PAs, 61 females (45.5%) and 73 males (54.5%). The median age was 34 years (range, 11-85 years), and the age distribution included patients in each decade of age between 10 and 90 years (Table 1). The PAs were 31 microadenomas (23.0%) and 103 macroadenomas (77.0%) with a diagnosis of 46 (34.3%) prolactinomas, 17 (12.7%) somatotroph PAs, 6 (4.5%) somatolactotroph PAs, 26 (19.4%) nonfunctioning PAs, 24 (17.9%) gonadotropinomas, 13 (9.7%) corticotroph adenomas, and 2 (1.5%) TSHomas (see Table 1). None of those patients had a family history of PA or a known PA-associated syndrome.

Variants in Pituitary Adenoma–Associated Genes

We detected 55 different nonsynonymous variants in 20 PA-associated genes in 52 patients (38.8%). Some of these variants occurred in more than one patient (Table 2, Supplementary Table S2) [14]. All of these variants except one (*ATRX*:c.A3815G, p.N1272S) are heterozygous (98.2%; see Supplementary Table S2) [14]. These variants were found in the following genes: *CDH23* (21 variants in 23 patients), *USP48* (2 variants in 9 patients), *GNAS* (4 variants in 6 patients), *MSH6* (4 variants in 5 patients), *SDHAF2* (2 variants in 5 patients), *SDHB* (2 variants in 4 patients), *ATRX* (3 variants in 3 patients), *SDHD*, *CDKN1B*, *MSH2*, *and NR3C1* (2 variants in 2 patients each), *MEN1* (1 variant in 2 patients), *AIP*, *DICER1*, *MLH1*, *NF1*, *PIK3CA*, *PMS2*, *SDHA*, and *USP8* (1 variant in 1 patient

each) (see Table 2). These variants included missense, nonsense, and small indels (see Supplementary Table S2) [14].

Assessment of pathogenicity of variants

Using the American College of Medical Genetics and Genomics (ACMG) classification [15], of the 55 variants found, 20 (36.3%) were benign or likely benign, and 35 (63.6%) were variants of unknown significance (VUS) (see Table 2 and Fig. 1). We then considered VUS for additional analysis.

To characterize VUS further, we assessed their frequencies in the 1000 Genomes and gnomAD databases in addition to their frequencies in the Saudi Genome Project (SGP) database. We initially used the ACMG designation of these variants as the primary method for predicting their pathogenicity [15]. We also used several in silico analysis tools and databases to assess variant pathogenicity, including ClinVar, MutationTaster, SIFT, PROVEAN, MutPred, PrimateAI, FATHMM, FATHMM-MKL, FATHMM-XF, MVP, and PolyPhen-2 (see Supplementary Table S2) [14].

As can be seen in Supplementary Table S2 [14], all of these VUS have extremely low frequencies or have never been reported before in international and local databases. The highest rates of VUS occurred in *CDH23* (12/35, 34.3%), *MSH6* (4/35, 11.4%), *SDHD*, *CDKN1B*, *NR3C1*, and *MSH2*, (each occurred in 2/35, 5.7%), and *SDHA*, *SDHB*, *AIP*, *ATRX*, *DICER1*, *NF1* and *PIK3CA* (each occurred in 1/35, 2.9%) (see Table 2).

ACMG criteria are generally strict and tend to err toward a non-pathogenic designation or VUS unless there is conclusive or compelling evidence of pathogenicity or likely pathogenicity. For this reason, we also analyzed data by a number of in silico analysis tools. Many of these VUS are predicted to be disease-causing or damaging by several in silico analysis tools. For example, using the recently released Alpha Missense (https://zenodo.org/records/8208688) [16], 7 ACMG-designated VUS and 1 that is designated benign are predicted to be likely pathogenic by the Alpha Missense analysis, including *AIP*:c.767C>T (p.S256F), *CDH23*:c.906G>C (p.E302D), *CDH23*:c.1096G>A (p.A366T), *DICER1*: c.620C>T (p.A207V), *MLH1*:c.955G>A (p.E319K), *MSH2*: c.148G>A (p.A50T), *SDHA*: c.869T>C (p.L290P) and *USP48*:c.2233G>A (p.V745M) occuring in 2 patients (Table 3 and Supplementary Table S2) [14]. These were also designated probably damaging by the commonly used and highly reliable PolyPhen2 and several other in silico tools (see Supplementary Table S2) [14]. Taken together, the very low rates or absence of these variants in the public databases including the patient population database (SGP) and the consistent likely pathogenic or probably damaging designation of these variants by Alpha Missense and PolyPhen 2 suggest the pathogenicity of these variants and their likely contributions in the PA pathogenesis in the patients that carry these variants. So, we focused on these 8 likely pathogenetic variants in our analysis and discussion.

Clinical Correlation

Focusing on the 8 likely pathogenic variants designated likely pathogenic by Alpha Missense analysis, the most common genes with variants were *CDH23* (2 variants). Each of the other genes (*AIP*, *DICER1*, *MLH1*, *MSH2*, *SDHA* and *USP48*) has one variant (see Table 3). The median age of patients

Table 1. Clinical features of 134 patients with pituitary adenomas

Characteristic	No.
Median age (range), y	34 (11-85)
Age groups	
<10	0
10.1-20	18 (13.4%)
20.1-30	39 (29.1%)
30.1-40	27 (20.1%)
40.1-50	25 (18.7%)
50.1-60	18 (13.4%)
60.1-70	5 (3.7%)
>70	2 (1.5%)
Sex (M:F)	73:61
PA type	
Microadenoma	31 (23.0%)
Macroadenoma	103 (77.0%)
Diagnosis	
Acromegaly	17 (12.6%)
Prolactinomas	46 (34.3%)
Somatolactotroph PA	6 (4.5%)
Nonfunctioning PA	26 (19.4%)
Gonadotrophinoma	24 (17.9%)
Cushing disease	13 (9.7%)
TSHoma	2 (1.5%)

Abbreviations: F, female; M, male; PA, pituitary adenoma; TSH, thyrotropin.

Table 2. Overall findings of variants in pituitary adenoma–associated genes, their frequency, and designation according to American College of Medical Genetics and Genomics criteria

No.	Gene	No. of patients[a]	No. of variants[b]	Benign	Likely benign	Unknown significance
1	*AIP*	1	1	0	0	1
2	*ATRX*	3	3	1	1	1
3	*CDH23*	23	21	1	8	12
4	*CDKN1B*	2	2	0	0	2
5	*DICER1*	1	1	0	0	1
6	*GNAS*	6	4	1	2	1
7	*MEN1*	2	1	0	0	1
8	*MLH1*	1	1	0	0	1
9	*MSH2*	2	2	0	0	2
10	*MSH6*	5	4	0	0	4
11	*NF1*	1	1	0	0	1
12	*NR3C1*	2	2	0	0	2
13	*PIK3CA*	1	1	0	0	1
14	*PMS2*	1	1	0	1	0
15	*SDHA*	1	1	0	0	1
16	*SDHAF2*	5	2	1	1	0
17	*SDHB*	4	2	1	0	1
18	*SDHD*	2	2	0	0	2
19	*USP8*	1	1	1	0	0
20	*USP48*	9	2	1	0	1
	Total	**52**[a]	**55**	**7**	**13**	**35**

[a]Some patients have multiple variants.
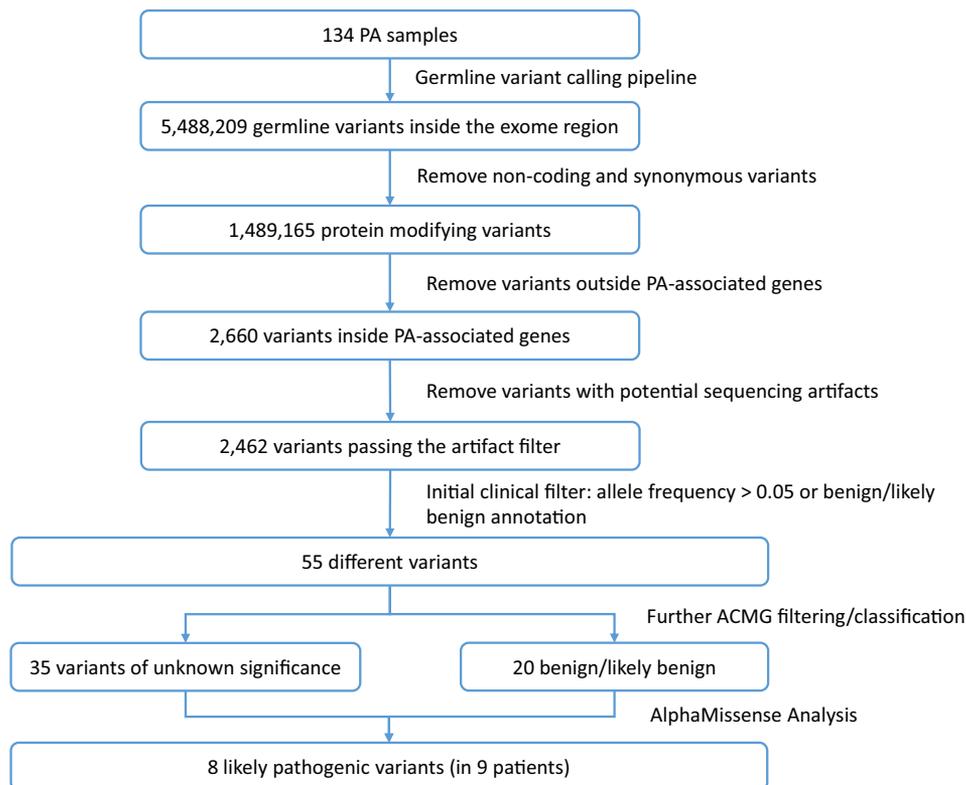[b]Some patients had additional variants in the same gene and/or other genes.

Figure 1. A flowchart illustrating the steps of the bioinformatics analysis of whole-exome sequencing of 134 patients with pituitary adenoma (PA) included in this study.

Table 3. Eight patients with likely pathogenic genetic variants in different types of pituitary adenomas according to Alpha Missense analysis tools

Gene	Transcript ID	Variant	Variant type	ACMG class	Alpha Missense class	Age at Dx, y	Sex	Diagnosis	Type, size
AIP	NM_003977	c.767C>T, p.S256F	Exonic, missense	VUS	Likely pathogenic	59	F	NF	Macro
CDH23	NM_022124	c.906G>C, p.E302D	Exonic, missense	VUS	Likely pathogenic	48	M	ACRO-PL	Macro
CDH23	NM_022124	c.1096G>A, p.A366T	Exonic, missense	Benign	Likely pathogenic	22	F	NF	Macro
DICER1	NM_001195573	c.620C>T, p.A207V	Exonic, missense	VUS	Likely pathogenic	21	F	PL	Macro
MLH1	NM_000249	c.955G>A, p.E319K	Exonic, missense	VUS	Likely pathogenic	63	M	GN	Macro
MSH2	NM_000251	c.148G>A, p.A50T	Exonic, missense	VUS	Likely pathogenic	55	M	PL	Macro
SDHA	NM_004168	c.869T>C, p.L290P	Exonic, missense	VUS	Likely pathogenic	48	M	ACRO-PL	Macro
USP48	NM_032236	c.2233G>A, p.V745M	Exonic, missense	VUS	Likely pathogenic	30	F	GN	Macro
USP48	NM_032236	c.2233G>A, p.V745M	Exonic, missense	VUS	Likely pathogenic	22	M	PL	Micro

Abbreviations: ACMG, American College of Medical Genetics and Genomics; ACRO, acromegaly; ACRO-PL, acromegaly-prolactinoma (somatolactotroph adenoma); CD, Cushing disease; Dx, diagnosis; F, female; GN, gonadotropinoma; M, male; Macro, macroadenoma; Micro, microadenoma; NF, nonfunctioning; PL, prolactinoma; VUS, variant of unknown significance.

was 48 years (range, 21-63 years) with 44.4% of them aged 30 years or younger. Four (44.4%) were females and 5 males. The PA was macroadenoma in all of them except one (microadenoma). Two cases were nonfunctioning PA, 2 macroprolactinoma, 1 microprolactinoma, 2 gonadotropinoma, and 2 mixed GH- and prolactin-secreting PAs (see Table 3).

Discussion

In this study, we report a high prevalence of nonsynonymous germline variants in PA-predisposing genes in patients with apparently sporadic PA, including genes previously known to be only somatically mutated in PA. This may potentially expand the underlying genetic profile of apparently sporadic PA. The high prevalence of putative underlying germline variants in our study, which was undertaken in patients without known family history of PA or PA-associated syndromes, suggests that the rate of germline variants might be even higher in those with FIPA. It may also reflect the high sensitivity of the NGS.

We have followed a rigorous bioinformatics analysis to increase the likelihood of the pathogenic nature of the genetic alterations we found and to minimize misinterpretation of nonpathogenic variants as pathogenic or likely pathogenic. We excluded low-quality amplicons and those with a variant allele frequency of less than 25% and focused on exons and splicing regions and genes previously associated with PA as germline, mosaic, or somatic genetic alterations. We initially followed the ACMG criteria in designating variants and excluded benign and likely benign variants. We also excluded variants with a minor allele frequency of greater than 0.05 in international databases but, more important, in our population genetic database of more than 13 000 individuals fully sequenced as part of the national genomic screening program

(SGP). As seen in Supplementary Table S2 [14], the variants we describe in this report were either not reported in these international or national databases or their minor allele frequency is extremely low. These features are strong indicators of the significance of these variants and lend credibility to our findings and suggest that these variants may play a part in the PA pathogenesis in these patients [17]. Using the recently released Alpha Missense tool showed that some of these variants designated as VUS by the strict ACMG criteria are likely pathogenic (see Table 3 and Supplementary Table S2) [14]. Alpha Missense is an artificial intelligence–based tool based on the Alpha Fold of protein and is viewed as one of the most important advances in the prediction model of genetic variants [16, 18, 19]. In a recent evaluation of 2073 variants from 686 patients with different hematological malignancy, Alpha Missense showed a very high accuracy in a large real-life data set of missense mutations with an area under the receiver operating characteristic curve of 0.95 [19]. The likely pathogenic germline variants found in the present study were in *AIP*, *CDH23*, *SDHA*, *DICER1*, *USP48*, *MSH2*, and *MLH1*. Some of these genes have well established roles in tumorogenesis of PA and other tumors (*AIP*, *SDHA*, *DICER1*, *MSH2*, and *MLH1*), and this supports their role in PA. Others (*CDH23* and *USP48*) also have fair evidence of their role in the pathogenesis of PA and will be discussed further later.

AIP is reportedly the most commonly mutated germline gene in FIPA, occurring in about 10% to 20% of FIPA but in less than 4% of apparently sporadic PAs [8]. The *AIP* gene encodes a 330-amino-acid co-chaperone involved in many cellular processes, including subcellular trafficking, nuclear receptor stability, and transactivation potential [8, 20, 21]. *AIP* mutations are inherited in an autosomal dominant pattern but have a low penetrance rate (∼20%). They are

most commonly associated with somatotroph or somatolactotroph adenomas occurring at a young age and are usually invasive and poorly responsive to somatostatin analogues [9, 22]. We found only one heterozygous missense *AIP* variant (NM_003977:c.767C>T, p.S256F) in a 59-year-old woman with nonfunctioning macroadenoma, negative for staining of all hormones, including GH and prolactin.

CDH23 is a cadherin superfamily member and is involved in cell-to-cell adhesion [23]. Therefore, mutations in this gene might promote tumor growth and invasion. We found 2 variants in this gene. The first variant, c.906G>C, changes glutamate to aspartate at codon 302. Although this has not been reported before, 2 variants in the preceding codon (p.R301W and p.R301Q) are designated pathogenic and are associated with hearing loss [24, 25]. This suggests that this region of the gene is important for its function and supports the pathogenicity of the c.906G>C variant. The second variant (c.1096G>A, p.A366T) is also a novel variant located between 2 previously reported pathogenic variants (p.Q362* and p.Q372*) causing Usher syndrome [26, 27]. Zhang et al [23] studied a large kindred with familial PA and found a heterozygous missense *CDH23* variant, c.4136G>T (p.Arg1379Leu), segregating in 4 affected and 17 asymptomatic individuals. To further ascertain the prevalence of *CDH23* variants in PA, these researchers screened 20 members of 12 additional families with FIPA, 125 individuals with sporadic PA, and 260 controls. This showed that 33% of the families with familial PA (4/12) and 12% of individuals with sporadic PA (15/125) harbored functional *CDH23* variants, but only 2 of 260 (0.8%) of the healthy control individuals carried functional *CDH23* variants. PA individuals who harbored *CDH23* variants had larger and more invasive PAs [23]. Therefore, our study supports the study by Zhang and colleagues [23] and suggests that this gene is a potential gene in the pathogenesis of PA.

The *SDHx* group encompasses 5 genes (*SDHA*, *SDHAF2*, *SDHB*, *SDHC*, and *SDHD*) that are the underlying genes of familial paragangliomas and pheochromocytoma (PPGL) syndromes. However, PAs have been rarely described in association with familial PPGL syndromes [28] and are sometimes referred to as the 3Pa syndrome [4]. Dénes et al [28] screened 39 patients with coexistent familial PPGL and PA (19 sporadic and 20 FIPA) for known PPGL genes (*SDHA-D*, *SDHAF2*, *RET*, *VHL*, *TMEM127*, *MAX*, and *FH*) and PA genes (*MEN1*, *AIP*, and *CDKN1B*). Eleven germline mutations were found, including 5 *SDHB*, 1 *SDHC*, 1 *SDHD*, 2 *VHL*, and 2 *MEN1*, in addition to 4 VUS in *SDHA*, *SDHB*, and *SDHAF2*. Using a custom 8-gene panel (*AIP*, *CDKN1B*, *MEN1*, *PRKAR1A*, *SDHA*, *SDHB*, *SDHC*, and *SDHD*), Sousa et al [29] studied 44 patients with PA. Fourteen of these 44 patients had a personal history of other endocrine tumors and/or a family history of pituitary or other endocrine tumors. Eleven patients harbored genetic variants, including variants in *SDHB* (p.A2V, p.S8S), *SDHC* (p.E110Q), and *SDHD* (p.G12S). We recently reported a young woman with a locally invasive recurrent macroprolactinoma resistant to cabergoline but responsive to temozolomide who had a pathogenic germline *SDHB* mutation [30]. In the present cohort with no personal or family history of PPGL, only the *SDHA* gene has one likely pathogenic variant in a patient with GH/prolactin-secreting macroprolactinoma (see Table 3).

The *DICER1* gene (14q32.13) encodes for a type III endoribonuclease that cleaves precursor microRNAs into mature microRNAs. These small noncoding RNAs regulate gene expression by targeting messenger RNA sequences for degradation [31]. Germline mutations in the *DICER1* gene are the underlying cause of *DICER1* syndrome, which has an early childhood onset and is characterized by an increased risk of pleuropulmonary blastomas, Sertoli-Leydig cell tumors, benign or malignant thyroid tumors, sarcomas, bony dysplasias, and pituitary blastomas [32, 33]. Pituitary blastoma is usually an ACTH-secreting macroadenoma associated with severe Cushing disease [34]. Recently, a pituitary microprolactinoma was diagnosed in a young woman with a *DICER1* mutation [35]. However, no further work was conducted to confirm the pathogenic role of the *DICER1* mutation in this microprolactinoma and it could have been a coincidental occurrence [35]. We found one likely pathogenic variant (NM_001195573, c.620C>T, p.A207V) in a 22-year-old woman with no significant personal or family history of other tumors/diseases. She had a 1.7-cm cystic macroprolactinoma with a plasma prolactin level of 212 μg/L (normal range, 3.4-24.1 μg/L). She responded well to cabergoline. Her prolactin normalized, and the PA decreased to 8 mm over 3 years.

Somatic mutations in *USP8* [36, 37] and *USP48* [38] have been reported in about 50% of corticotroph adenomas. Although *USP8* mutations are more common, *USP48* mutations occur in about 13% of cases of Cushing disease and involve mostly a hot spot at p.Met415 [38, 39]. USP8 and USP48 are deubiquitinases that lead to reversal of ubiquitination, a process that is important for proteasomal degradation, transcriptional regulation, and protein trafficking [39]. Mutations in *USP8* and *USP48* increase the deubiquitination leading to recycling of many proteins, the best known of which is EGFR for USP8 and Gli1 and histone H2A for USP48 [40]. Deubiquitination of Gli1 increases the SHH pathway activity resulting in ACTH activation and deubiquitination of H2A promote PA growth [40]. Therefore, mutations in *USP8* and *USP48* lead to activation of the enzymatic activity of these molecules culminating in activation of pathways that lead to tumor development and growth. A *USP8* germline mutation has been described in a young girl who had dysmorphic features, developmental delay, and multiple organ dysfunction. She developed Cushing disease at age 14 years [41]. So far, no germline *USP48* mutations have been described. In this study, we found 1 likely pathogenic variant in *USP48* (NM_032236:c.2233G>A, p.V745M) in a 30-year-old woman with a gonadotroph adenoma, positive for LH/FSH but negative for ACTH staining, and in a 21-year-old man with microprolactinoma.

Finally, *MLH1*, *MSH2*, and *MSH6* are some of the underlying genes for Lynch syndrome (LS), also known as hereditary nonpolyposis colorectal cancer (HNPCC) [42, 43]. LS is characterized by an increased risk of developing several types of cancers due to germline mutations in genes involved in DNA mismatch repair (*MSH2*, *MLH1*, *MSH6*, *PMS2*, and *EPCAM*) [42]. Colorectal, endometrial, ovarian, gastric cancers and malignant brain tumors are among the commonly occuring cancers seen in this syndrome [42, 43]. PA has been associated with LS, and mutations in LS genes have been described in apparently sporadic PA [44-46]. In our study, one *MSH2* and one *MLH1* likely pathogenic variants were found in a 55-year-old man with macroprolactinoma and a 63-year-old man with gonadotropinoma, respectively. Neither of these patients had a personal or family history of LS.

This study contributes to our understanding of the germline genetics of PA and brings some novel findings to this field. It

shows that germline variants in genes that were reported before to be mutated in germline, mosaic, or somatic patterns are not uncommon. Although most of these variants are VUS, their in silico analysis and low frequencies in genetic variant databases suggest potential pathogenic roles in PA. Furthermore, about 14.5% (8/55) of these variants were found to be likely pathogenic by the recently released highly reliable Alpha Missense analysis tool [16]. These higher percentages than previously described might be due to the use of more sensitive methods of detection (NGS). The effect of population structure might also be a factor since there is a high rate of consanguinity in the Saudi population [47, 48]. However, considering that the vast majority of variants found in this study are heterozygous, the effect of consanguinity may be minor.

This study has some limitations, including a lack of functional characterization of the variants and family segregation studies. Excluding patients with a family history of PA or PA-associated syndromes has also decreased the chance of finding pathogenic variants but this has also resulted in a more homogeneous sample of PAs. We also limited our search for genetic variants to genes that were previously reported to be associated with PA in germline, mosaic, or somatic forms. This may have limited discovery of novel genetic associations. Future studies are needed to confirm our findings and address these limitations.

In summary, pending further confirmatory studies, this study suggests that about 7% of patients (9/134) with apparently sporadic PAs may have likely pathogenetic germline variants in well-known genes, including some genes that were previously known to be only somatically mutated in PAs. VUS occur in an additional 23.8% of patients (32/134) and need further studies to evaluate their significance.

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Disclosures

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Data Availability

Some or all data sets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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