ARMC5 Mutations Are a Frequent Cause of Primary Macronodular Adrenal Hyperplasia

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Context: Primary macronodular adrenal hyperplasia (PMAH) is a rare cause of Cushing's syndrome, usually characterized by functioning adrenal macronodules and increased cortisol production. Familial clustering of PMAH has been described, suggesting an inherited genetic cause for this condition.

Objective: The aim of the present study was to identify the gene responsible for familial PMAH.

Patients and Methods: Forty-seven individuals of a Brazilian family with PMAH were evaluated. A single-nucleotide polymorphism-based genome-wide linkage analysis followed by whole-exome sequencing were then performed in selected family members. Additionally, 29 other patients with PMAH and 125 randomly selected healthy individuals were studied to validate the genetic findings. Moreover, PMAH tissue was also analyzed through whole-exome sequencing, conventional sequencing, and microsatellite analysis.

Results: A heterozygous germline variant in the *ARMC5* gene (p.Leu365Pro) was identified by whole-exome sequencing in a candidate genomic region (16p11.2). Subsequently, the same variant was confirmed by conventional sequencing in all 16 affected family members. The variant was predicted to be damaging by in silico methods and was not found in available online databases or in the 125 selected healthy individuals. Seven additional *ARMC5* variants were subsequently identified in 5 of 21 patients with apparently sporadic PMAH and in 2 of 3 families with the disease. Further molecular analysis identified a somatic mutational event in 4 patients whose adrenal tissue was available.

Conclusions: Inherited autosomal dominant mutations in the *ARMC5* gene are a frequent cause of PMAH. Biallelic inactivation of *ARMC5* is consistent with its role as a potential tumor suppressor gene. **(*J Clin Endocrinol Metab* 99: E1501–E1509, 2014)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received November 27, 2013. Accepted March 28, 2014.
First Published Online April 7, 2014

Abbreviations: ARMC5, armadillo repeat-containing 5; CS, Cushing's syndrome; CT, computed tomography; CV, coefficient of variation; DST, dexamethasone suppression test; NR, normal range; PMAH, primary macronodular adrenal hyperplasia; SNP, single-nucleotide polymorphism.

Primary macronodular adrenal hyperplasia (PMAH), also known as ACTH-independent macronodular adrenal hyperplasia, bilateral macronodular adrenal hyperplasia, massive macronodular adrenocortical disease, autonomous macronodular adrenal hyperplasia, ACTH-independent massive bilateral adrenal disease, giant or huge macronodular adrenal hyperplasia, and macronodular adrenal dysplasia, is a rare cause of Cushing's syndrome (CS), accounting for less than 2% of all endogenous CS cases (1–4). First described by Kirschner et al in 1964 (5), PMAH is usually characterized by functioning adrenal macronodules and increased cortisol production. The sporadic form of the disease appears to be the most frequent; however, the true prevalence of the familial form is unknown. Reports of familial clustering have been described in recent years (6–17), suggesting that the disease may be inherited in an autosomal dominant manner.

Despite being a known clinical entity for almost 50 years, the pathophysiological process that leads to PMAH and the predisposing genetic alterations underlying the disease have not been fully clarified. The mechanism by which cortisol production is stimulated in PMAH, despite suppressed plasma ACTH, is complex. It has been previously demonstrated that cortisol secretion in PMAH may be regulated by hormones other than ACTH, via aberrant expression of G protein-coupled hormone receptors in the adrenal glands (18, 19). However, it is not totally clear whether the expression of these receptors is the primary phenomenon responsible for PMAH pathogenesis or a secondary phenomenon resulting from cell proliferation and dedifferentiation. Furthermore, no mutations have been found in the coding or promoter regions of aberrant hormone receptor genes (20). Recently, it was found that cortisol secretion in PMAH may be regulated by ACTH, which is produced by a subpopulation of steroidogenic cells in the hyperplastic adrenals (autocrine/paracrine regulatory mechanisms) (3). The abnormal expression of ACTH by these adrenocortical cells seems to represent a secondary pathophysiological process that is common to diverse molecular defects and is seen in both sporadic and familial cases (3). Based on this important finding, ACTH-independent macronodular adrenal hyperplasia is no longer an appropriate name for the disease and therefore, in this manuscript the disease has been termed primary macronodular adrenal hyperplasia (PMAH).

The hypothesis of activating mutations in the ACTH receptor (*MC2R*) leading to the constitutive secretion of cortisol has also been investigated in PMAH, with only 1 case described in the literature (21). Alterations in the signaling pathways downstream of the ACTH receptor have also been evaluated. Somatic mutations in the gene encoding the Gsα subunit of heterotrimeric G protein (*gsp* mutations) were found in a few patients with PMAH in the absence of the typical clinical features of McCune-Albright syndrome (22, 23). The real role of these somatic mutations in the pathophysiological process of the disease still needs to be clarified. PMAH has also been reported in patients with multiple endocrine neoplasia type 1 syndrome, familial adenomatous polyposis, and hereditary leiomyomatosis and renal cell cancer disorder (24–26). Nevertheless, mutations in genes associated with these syndromes (*MEN1, APC*, and *FH,* respectively) have not been described in PMAH outside of the context of these genetic disorders. More recently, inactivating germline and somatic mutations in the armadillo repeat-containing 5 (*ARMC5*) gene, a putative tumor-suppressor gene, were reported in patients with PMAH (27).

In this study, we sought to identify the genetic cause of PMAH by performing a single-nucleotide polymorphism (SNP)-based genome-wide linkage study and a whole-exome sequence analysis of a large family with PMAH.

Patients and Methods

Patients

A total of 47 patients from a large family with PMAH were initially selected for the present study (Figure 1). Each patient underwent clinical, laboratory, and radiological assessments for the presence of PMAH and CS. Initial evaluation consisted of a medical history, a detailed physical examination, a 1-mg overnight dexamethasone suppression test (DST), a late-night salivary cortisol measurement, and an adrenal computed tomography (CT) scan. Any laboratory abnormality initially observed was subsequently confirmed in a second test; at this time, plasma dexamethasone was also measured on the DST. The diagnosis of PMAH was established in patients who presented both increased serum cortisol levels (>1.8 μg/dL or >50 nmol/L) after the DST and radiological features compatible with the disease. In these cases, a subsequent evaluation was then carried out to assess 24-hour urinary cortisol and plasma ACTH levels.

In addition, 29 other patients with PMAH followed at Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo in Brazil (21 apparently sporadic cases and 8 familial cases from 3 other families) and 125 healthy controls were selected for validation purposes. Fresh-frozen adrenal tissue was also obtained from 4 patients with PMAH who underwent an adrenalectomy (2 members of the largest family [individuals IV-5 and IV-11] and 2 nonrelated patients) for further genetic studies (Figure 1).

The study was approved by the Ethics Committee of São Paulo University, and written informed consent was obtained from all participants.

Hormone assays

Serum cortisol was measured by a chemiluminescent immunoassay (Immulite 2000; Siemens Healthcare Diagnostics Inc) with an intra-assay coefficient of variation (CV) of 6.0%, inter-assay CV of 7.8%, and normal range (NR) ≤1.8 μg/dL (≤50

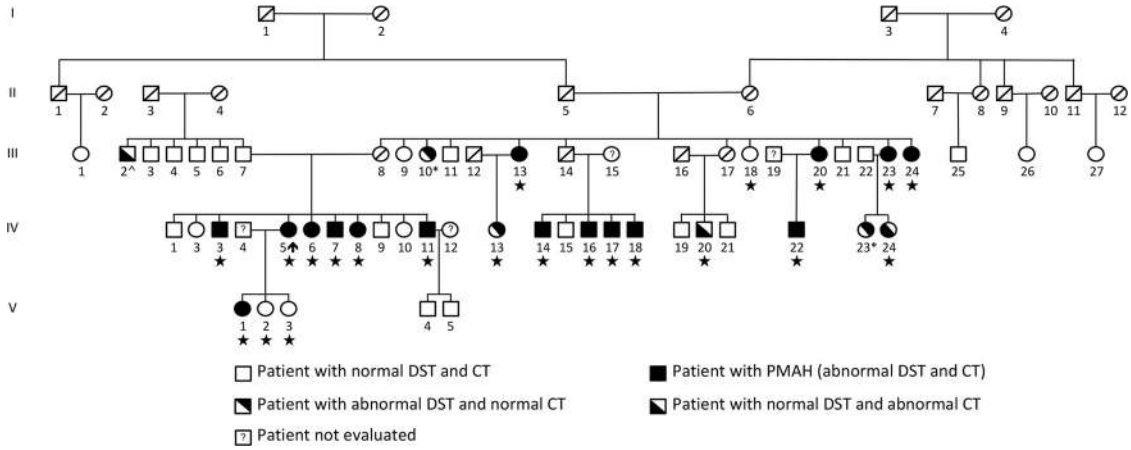

Figure 1. Pedigree of the family with familial PMAH. Squares indicate male family members, circles indicate female family members, and symbols with a diagonal line indicate deceased family members. The stars indicate family members with a heterozygous germline mutation in the *ARMC5* gene, and an arrow points to the proband. All patients (family members) with PMAH showed abnormal DST results (serum cortisol >1.8 μg/dL or >50 nmol/L, and plasma dexamethasone ≥180 ng/dL) and radiological findings compatible with the disease (thickened and/or nodular adrenals). ^, A single nodule of 1 cm was discovered in the left adrenal gland of patient III-2 during the radiological investigation. *, Plasma dexamethasone levels were <180 ng/dL on DST in patients III-10 and IV-23.

nmol/L) after DST. The late-night salivary cortisol was measured by a RIA (Coat-A-Count; Siemens Healthcare Diagnostics) with an intra-assay CV of 4.8%, interassay CV of 5.2%, and NR ≤0.13 μg/dL (≤3.6 nmol/L). Plasma dexamethasone levels were determined by liquid chromatography-tandem mass spectrometry (Quest Diagnostics Nichols Institute) with NR ≥180 ng/dL on DST. Total urinary cortisol (unconjugated and conjugated cortisol) was measured by a chemiluminescent immunoassay (Immulite 2000; Siemens Healthcare Diagnostics) with an intra-assay CV of 5.3%, interassay CV of 7.5%, and NR 50–310 μg/24 hours (138–855.6 nmol/24 hours). The urinary free cortisol was measured after dichloromethane extraction by a chemiluminescent immunoassay (Unicel DxI 800; Beckman Coulter Inc) with an intra-assay CV of 6.7%, interassay CV of 7.9%, and NR 21–111 μg/24 hours (58–306.4 nmol/24 hours). Plasma ACTH was determined by a chemiluminescent immunometric assay (Immulite 2000; Siemens Healthcare Diagnostics) with an intra-assay CV of 7.7%, interassay CV of 8.5%, and NR 10–46 pg/mL (2.2–10.2 pmol/L).

Adrenal CT scans

Unenhanced multislice CT scans were acquired from all 47 patients initially evaluated for familial PMAH, and 3-mm-thick sections through both adrenal glands were obtained. Measurements of maximum adrenal body and limb widths were taken, and the size and attenuation of the nodules were noted using the electronic cursor function. The adrenal glands were defined as thickened if at least 2 of their elements were enlarged (body width ≥12.5 mm and limb width ≥5.5 mm), similarly to previous studies (28, 29). All adrenal images were available in DICOM format and were reviewed independently by 2 radiologists who were unaware of the clinical features and laboratory findings of the patients.

Molecular studies

SNP-based genome-wide linkage analysis

Genomic DNA was extracted from peripheral blood leukocytes by a salting-out procedure, according to standard protocols (30). An SNP-based genome-wide linkage study was performed at Prognomix at Centre de Recherche du Centre Hospitalier de l'Université de Montréal (Montreal, Quebec, Canada) using the Affymetrix Genome-Wide Human SNP Array version 6.0 (Affymetrix Inc, Santa Clara, CA). A total of 15 family members with PMAH (patients III-20, III-23, III-24, IV-3, IV-5, IV-6, IV-7, IV-8, IV-11, IV-14, IV-16, IV-17, IV-18, IV-22, and V-1) and 9 nonaffected controls (patients III-1, III-9, III-18, III-25, III-26, III-27, IV-1, IV-3, and IV-19) were enrolled in this analysis (Figure 1). To avoid misclassifying the patients, given the late onset of PMAH, only controls older than 60 years were included. The bioinformatics analysis was conducted using Statistical Analysis for Genetic Epidemiology (S.A.G.E.) software, version 6.1.0 (Case Western Reserve University, Cleveland, OH). Model-free linkage analysis was performed using the SIPBAL and LODPAL programs. Model-based linkage analysis using the LODLINK program was performed under the assumption of an autosomal dominant mode of inheritance and a penetrance of 90%. A *P* value and/or an LOD score were calculated for each SNP genotyped, and thereby candidate genomic regions suspected of harboring the PMAH susceptibility locus were identified (for details, see Supplemental Methods).

Whole-exome sequencing analysis

Whole-exome sequencing was performed next at Personal Genome Diagnostics Inc using the Illumina HiSeq 2000 platform (Illumina Inc). Six patients with PMAH (III-20, III-23, IV-5, IV-11, IV-14, and IV-17) and 4 nonaffected controls (III-7, III-9, III-18, and IV-I) were enrolled in this subsequent analysis (Figure 1). We searched for germline variants that occurred in all affected patients (cases) but were absent in most controls. Due to the possibility of an incomplete penetrance of the disease or to a very late onset, the presence of asymptomatic carriers was expected. Moreover, a variant found within a suspected genomic region identified by the linkage analysis was also more likely to be associated with the disease (see Supplemental Methods for details).

Genomic DNA was also extracted from hyperplastic adrenal glands according to standard protocols (see Supplemental Methods for details). To identify somatic mutational events, whole-

exome sequencing analysis was also performed on the adrenal samples from 2 patients (individuals IV-5 and IV-11) (see Supplemental Methods for details).

Validation studies

ARMC5 variants and loss-of-heterozygosity events identified by whole-exome sequencing were confirmed by the Sanger sequencing method and a microsatellite analysis, respectively (see Supplemental Methods for details). The *ARMC5* gene was further sequenced by the Sanger method in 29 other patients with PMAH (Supplemental Table 1). The functional impacts of the variants detected herein were predicted by 4 different in silico analyses: PolyPhen (http://genetics.bwh.harvard.edu/pph) (31), SIFT version 2 (http://sift.jcvi.org) (32), MutationTaster (http://www.mutationtaster.org) (33), and SNAP (http://rostlab.org/services/snap) (34).

Results

Diagnosis of familial PMAH

In the largest studied family, 47 patients of white ethnicity were evaluated for the presence of PMAH (Figure 1 and Supplemental Table 2). Familial PMAH was diagnosed in 16 patients based on laboratory features and radiological findings. The mean age at diagnosis was 54.8 ± 13.4 years (ranging from 32–84 years). All patients with PMAH showed abnormal DST results (serum cortisol >1.8 μg/dL or >50 nmol/L; and plasma dexamethasone ≥180 ng/dL) and radiological findings compatible with the disease (thickened and/or nodular adrenals) (Table 1). In nearly 38% (6 of 16) of the affected family members (chronological ages from 32–84 years), only 1 adrenal gland was enlarged (thickened and/or nodular) on CT scan at diagnosis. Most of the affected patients exhibited only a few subtle signs and symptoms of CS, normal midnight salivary and 24-hour urinary cortisol, and normal or only partially suppressed ACTH levels (5–10 pg/mL or 1.1–2.2 pmol/L) (Table 1). In some patients with PMAH, the signs and symptoms of CS were absent.

PMAH occurred in 3 consecutive generations, equally affecting men and women (50% of each sex), and it could be passed down by both sexes. Furthermore, approxi-

Table 1. Laboratory Features and Radiological Findings in Patients With Familial PMAH[a]

Patient	Sex	Age, y	Serum Cortisol, μg/dL[b]		Plasma Dexamethasone on 2nd DST, ng/dL[c]	Late-Night Salivary Cortisol, μg/dL[d]		Urinary Cortisol, μg/24 h[e]		Plasma ACTH, pg/mL[f]	Adrenal CT
			After First DST	After Second DST		First Test	Second Test	Total	Free		
III-13	F	84	**4.0**	**5.1**	457	**0.25**	—	—	—	—	L Ad: normal R Ad: thickened
III-20	F	74	**19.1**	**25.6**	180	**0.16**	—	—	91	**5.0**	L Ad: thickened with nodules R Ad: thickened with nodules
III-23	F	70	**12.0**	**10.5**	681	<0.10	<0.10	78	—	**5.2**	L Ad: thickened with nodules R Ad: thickened with a nodule
III-24	F	67	**3.7**	**1.9**	182	<0.10	<0.10	121	51	18.3	L Ad: thickened with a nodule R Ad: with a nodule
IV-3	M	59	**6.2**	**11.1**	715	**0.26**	0.11	85	73	13.0	L Ad: thickened with nodules R Ad: thickened with nodules
IV-5	F	52	**19.7**	**15.8**	463	**0.18**	**0.18**	**521**	—	< **5.0**	L Ad: thickened with nodules R Ad: thickened with nodules
IV-6	F	55	**1.9**	**2.2**	378	<0.10	<0.10	—	36	16.7	L Ad: normal R Ad: with a nodule
IV-7	M	54	**3.3**	**3.3**	371	<0.10	<0.10	72	50	13.0	L Ad: normal R Ad: with a nodule
IV-8	F	53	**2.9**	**2.6**	283	<0.10	<0.10	90	—	—	L Ad: thickened with a nodule R Ad: with a nodule
IV-11	M	45	**15.0**	—	—	—	—	**374**	—	< **5.0**	L Ad: thickened with nodules R Ad: thickened with nodules
IV-14	M	49	**5.7**	**4.6**	256	<0.10	<0.10	304	109	**8.0**	L Ad: thickened with nodules R Ad: thickened with nodules
IV-16	M	46	**3.1**	**2.1**	271	<0.10	<0.10	168	50	10.0	L Ad: thickened with nodules R Ad: normal
IV-17	M	45	**4.0**	**2.9**	181	<0.10	<0.10	138	52	—	L Ad: thickened with nodules R Ad: thickened with nodules
IV-18	M	39	**3.8**	**3.3**	184	0.12	<0.10	106	59	—	L Ad: with a nodule R Ad: with a nodule
IV-22	M	52	**3.7**	**3.7**	309	<0.10	0.11	—	—	—	L Ad: thickened with a nodule R Ad: normal
V-1	F	32	**2.5**	**3.7**	209	<0.10	<0.10	62	58	28.0	L Ad: normal R Ad: thickened with nodules

Abbreviations: F, female; L Ad, left adrenal; M, male; R Ad, right adrenal.

[a] Abnormal laboratory data are highlighted in bold. —, Data not available.

[b] Serum cortisol after DST, NR ≤1.8 μg/dL (≤50 nmol/L).

[c] Plasma dexamethasone on DST, NR ≥180 ng/dL.

[d] Late-night salivary cortisol, NR ≤0.13 μg/dL (≤3.6 nmol/L).

[e] Total urinary cortisol, NR 50–310 μg/24 h (138–855.6 nmol/24 h); urinary free cortisol, NR 21–111 μg/24 h (58–306.4 nmol/24 h).

[f] Plasma ACTH, NR 10–46 pg/mL (2.2–10.2 pmol/L).

mately half of the siblings were affected in some segments of the pedigree, and no history of consanguinity could be established. Together, these findings favor the hypothesis of an autosomal dominant inheritance pattern.

Molecular studies

SNP-based genome-wide linkage analysis of the family with PMAH pointed toward potential susceptibility loci on chromosomes 16 and 11. Three candidate genomic regions (16p11.2, 16q13, and 16q21) were independently delineated by all 3 different linkage analysis methods (SIPBAL, LODPAL, and LODLINK) and therefore became the main regions suspected of harboring the PMAH susceptibility locus. Three other genomic regions (16p12.1, 16q12.1, and 11q23.1) were also delineated, but only by 2 different methods of analysis (Figure 2 and Supplemental Tables 3–5).

Whole-exome sequencing was then performed in 10 selected family members (6 cases and 4 unaffected members). An average of 24 104 germline sequence alterations were initially identified in each patient. Rigorous criteria were then used to filter the variants and identify the mutation likely to be causative of familial PMAH. We first searched for heterozygous variants that occurred in all affected patients (family cases) but were absent in most controls (unaffected family members); a total of 82 variants met this initial criterion (Supplemental Table 6). Subsequently, synonymous (silent) variants were filtered out, and only 48 variants remained to be analyzed. Finally, we excluded all variants with a minor allele frequency ≥1% in either the 1000 Genome database or in the dbSNP database. This analysis yielded a single heterozygous variant in the armadillo repeat containing 5 (*ARMC5*) gene (c.1094T→C). Importantly, the *ARMC5* gene is located in a genomic region (16p11.2) suspected to be associated with PMAH according to previous linkage analysis results (Figure 2). This *ARMC5* missense variant (p.Leu365Pro) was then confirmed by the Sanger sequencing method in all 16 affected family members. The p.Leu365Pro variant was absent in most unaffected family members (except 3 asymptomatic carriers) and in the 125 unrelated healthy Brazilian controls (Figure 1). Furthermore, this variant was predicted as damaging (nonneutral) by all 4 prediction tools (MutationTaster, Polyphen, SIFT, and SNAP) (31–34).

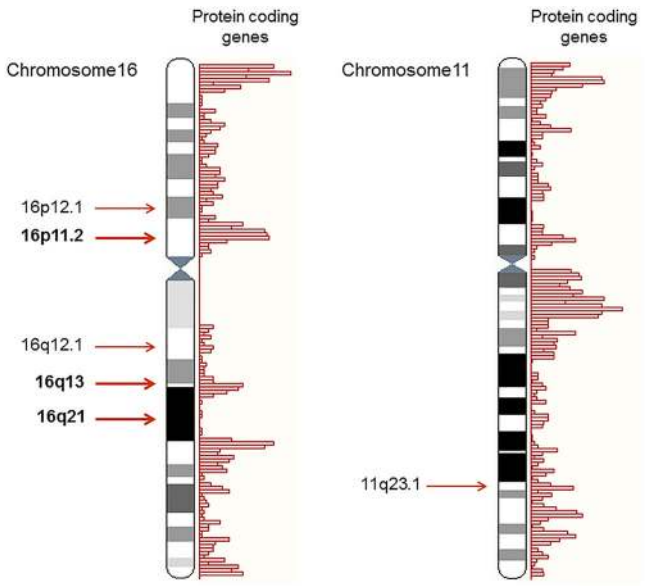

Figure 2. Candidate genomic regions suspected of harboring the PMAH susceptibility locus based on linkage analysis. The main suspected genomic regions (16p11.2, 16q13, and 16q21) were independently delineated by all 3 different linkage analysis methods (SIPBAL, LODPAL, and LODLINK) and are highlighted in bold. The other 3 suspected genomic regions (16p12.1, 16q12.1, and 11q23.1) were delineated by only 2 different methods of analysis. The histogram shows the density of protein-coding genes in each genomic region.

Whole-exome sequencing of DNA extracted from hyperplastic adrenals identified a somatic missense variant (p.Cys657Trp) and copy-neutral loss of heterozygosity of the entire 16p locus in patients IV-5 and IV-11, respectively (Figures 1 and 3; Supplemental Table 7 and Figure 1). These somatic mutational events were subsequently confirmed by Sanger sequencing and microsatellite analysis, respectively.

Based on the above-mentioned findings, 29 other patients with PMAH (21 apparently sporadic cases and 8 familial cases from 3 other families) followed at Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo in Brazil were studied to determine whether they carried *ARMC5* mutations. Nearly 29% of these patients (5 of 21 apparently sporadic cases and 2 of 3 index cases) showed germline *ARMC5* mutations. Additional somatic mutations were also found in the other 2 patients whose adrenal tissue was available (Table 2 and Figure 4).

Discussion

Despite being a known clinical entity for almost 50 years, the molecular pathogenesis of PMAH has remained largely unknown. Based on the study of the largest family so far described with the disease, we were able to identify a common missense mutation in the *ARMC5* gene in all affected family members. Therefore, a germline mutation has consistently been associated with familial PMAH.

The role of *ARMC5* mutations as a causative factor in PMAH is supported by distinct and complementary information provided by various bioinformatics and molecular tools. The whole-exome sequencing analysis initially revealed that a heterozygous germline mutation in the *ARMC5* gene (c.1094T→C) was the unique variant (not

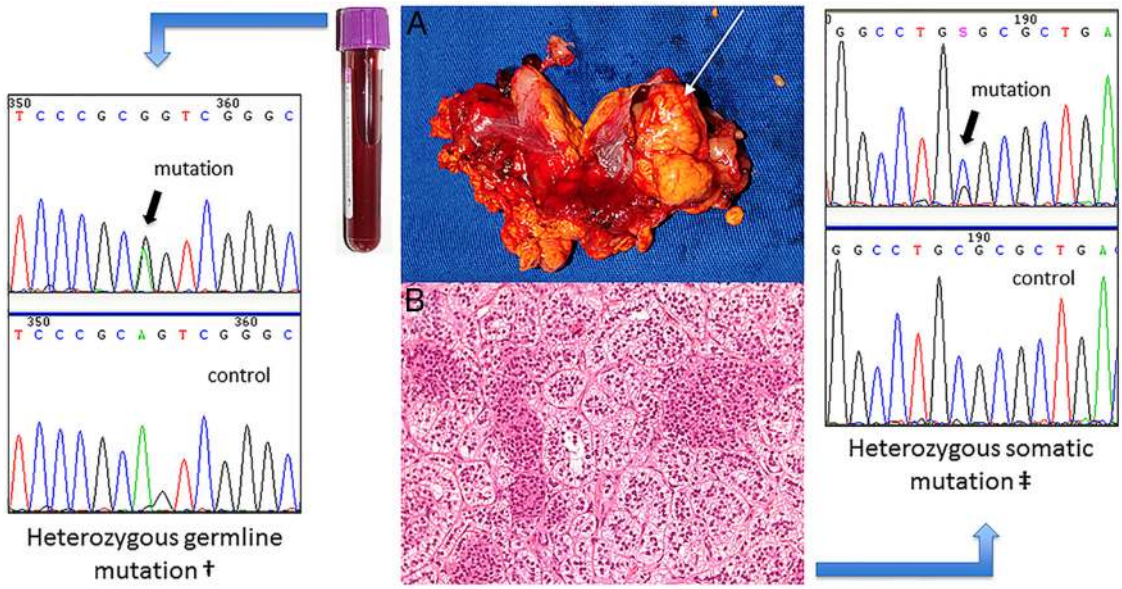

Figure 3. Germline and somatic mutations (p.Leu365Pro and p.Cys657Trp, respectively) in the *ARMC5* gene found in the index case (patient IV-5) of the largest family with PMAH. A, Macroscopic view of the resected right adrenal gland with yellowish nodules of various sizes. B, Histological view of resected adrenal tissue showing the presence of large cortical cells with clear cytoplasm (lipid-rich) forming string-like structures and small cells with compact cytoplasm (lipid-poor) forming island-like structures, shown by hematoxylin and eosin staining. †, Antisense strand sequencing; ‡, sense strand sequencing.

considered a polymorphism) present in all family members with PMAH and absent in most unaffected family members. This mutation was located in a genomic region predicted to be associated with PMAH according to all linkage analysis methods (SIPBAL, LODPAL, and LODLINK) and was predicted as damaging by all the prediction tools applied (MutationTaster, Polyphen, SIFT, and SNAP). In addition, other germline *ARMC5* mutations were subsequently detected by the Sanger sequencing method in nearly 24% of the patients (5 of 21) who were initially thought to have the sporadic form of the disease, demonstrating that the inherited form is much more common than previously believed. Furthermore, 2 additional families with PMAH harboring different germline mutations in the *ARMC5* gene were also identified. These findings are in accordance with those recently published by Assié et

Table 2. *ARMC5* Mutations Were Found in 27 Patients With PMAH[a]

			ARMC5 Gene[b]						
Patients With PMAH	**Sex**	**Clinical Manifestation**	**Alteration**	**DNA**	**Protein**	**MutationTaster[c]**	**PolyPhen[d]**	**SIFT[e]**	**SNAP[f]**
Family A, 16 patients	8 F, 8 M	Clinical and subclinical CS	Germline	c.1094T→C	p.Leu365Pro	+ (0.999)	+ (0.994)	+ (0.000)	+ (2)
Patient IV-5	F	Clinical CS	Somatic	c.1971C→G	p.Cys657Trp	+ (0.999)	+ (1.000)	+ (0.000)	+ (4)
Patient IV-11	M	Clinical CS	Somatic	del (16p)					
Family B, 4 patients	2 F, 2 M	Clinical and subclinical CS	Germline	c.2423A→C	p.His808Pro	+ (0.974)	+ (0.999)	+ (0.010)	+ (3)
Index patient	F	Clinical CS	Somatic	c.247_256del	p.Ala83Argfs*51	+ (1.000)			
Family C, 2 patients	2 F	Clinical and subclinical CS	Germline	c. (164_171)insG	p.Ile58Asnfs*45	+ (1.000)			
Patient D	F	Subclinical CS	Germline	c.2336C→G	p.Ser779*	+ (0.999)			
			Somatic	c.290_294del	p.Ala97Glyfs*4	+ (1.000)			
Patient E	F	Clinical CS	Germline	c.952C→G	p.Leu318Val	− (0.929)	+ (0.748)	− (0.260)	− (7)
Patient F	F	Clinical CS	Germline	c.1181T→C	p.Leu394Pro	+ (0.999)	+ (0.994)	+ (0.000)	+ (1)
Patient G	F	Clinical CS	Germline	c.1158G→A	p.Trp386*	+ (1.000)			
Patient H	F	Clinical CS	Germline	c.476–1G→C		+ (1.000)			

Abbreviations: F, female; M, male.

[a] Variant is predicted to be damaging by the bioinformatics tool (+); variant is predicted to be neutral by the bioinformatics tool (−).

[b] *ARMC5* gene Ensembl gene transcript identification ENST00000268314 and Ensembl protein identification ENSP00000268314 (UniProt peptide Q96C12).

[c] The score (in parentheses) is the probability of the prediction; a value close to 1 indicates a high security of the prediction.

[d] The score (in parentheses) represents the probability that a substitution is damaging; a value nearer 1 is more confidently predicted to be deleterious.

[e] The score (in parentheses) is the normalized probability that the amino acid change is tolerated; scores nearer 0 are more likely to be deleterious.

[f] The reliability index measure (in parentheses) correlates with the accuracy of the prediction (range 0–9); higher reliability values indicate a higher accuracy of the prediction.

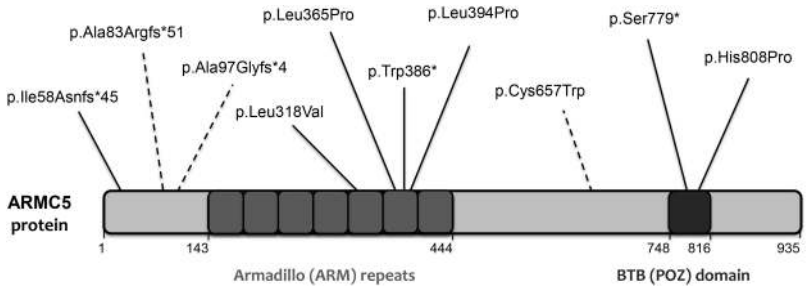

Figure 4. Schematic representation of the ARMC5 protein demonstrating germline (solid lines) and somatic (dotted lines) mutations. Ensembl protein identification ENSP00000268314 (UniProt peptide Q96C12).

al (27). By using a different approach, they have found germline *ARMC5* mutations in 56% of the patients with PMAH (14 of 25 patients). The *ARMC5* mutations identified in the present study were distributed all along the gene (Figure 4); however, they are different from those already described (27).

Our findings confirm the previous observations regarding the transmission pattern of PMAH, which were consistent with autosomal dominant inheritance (15–17). A second somatic mutational event (either a second point mutation or the loss of heterozygosity of the *ARMC5* locus) was also found in 4 patients whose adrenal tissue was available, which is in agreement with what has been reported recently (27). In functional studies performed by Assié et al (27), the *ARMC5* gene was capable of inducing apoptosis in H295R adrenal carcinoma cell lines; moreover, *ARMC5* inactivation was apparently associated with a slow process of dedifferentiation of adrenocortical cells, along with reduced expression of genes encoding steroidogenic enzymes and the melanocortin 2 receptor (MC2R) (27). All these findings suggest that *ARMC5* might act as a tumor suppressor gene, in accordance with Knudson's 2-hit model. The *ARMC5* gene is ubiquitously expressed in normal human tissues (http://www.genecards.org). In a preliminary investigation, we found typical images of intracranial meningiomas or a previous history of these tumors in 43% of the family members (3 of 7 evaluated patients) with germline *ARMC5* mutations and PMAH (patients IV-5, IV-6, and IV-16). In contrast, meningiomas are incidentally diagnosed in only 0.9% of the general population after the fourth decade of life (35). At the diagnosis of meningioma, patient IV-5 was 53 years old and had overt CS; patient IV-16 was 48 years old and had subclinical CS. In the patient IV-6, the meningioma occurred when she was 26 years old, 29 years before the diagnosis of subclinical CS and PMAH. Intracranial meningiomas have also been previously described in 2 other siblings with familial PMAH, but in these cases, no investigation for *ARMC5* mutations was carried out (13). Further studies are needed to investigate the potential association between *ARMC5* mutations and the occurrence of meningiomas and other tumors.

The specific function of the ARMC5 protein and its associated signaling pathways are still unknown. ARMC5 belongs to a large family of proteins known as armadillo repeat proteins. These proteins contain a repeating ~42–amino-acid motif, the armadillo (ARM) repeats (Figure 4), and are involved in development, the maintenance of tissue integrity, and tumorigenesis, among many other functions (36). Two proteins containing ARM repeats, β-catenin and adenomatous polyposis coli (APC), play important roles in the canonical WNT pathway (36, 37). The activation of this signaling pathway has already been associated with adrenal tumorigenesis and with PMAH pathophysiology (37–39). Further studies are needed to determine whether *ARMC5* also plays a role in the WNT pathway or in other signaling pathways.

Nearly 88% (14 of 16) of the patients diagnosed with PMAH in the largest studied family exhibited subclinical CS or only a few subtle signs and symptoms of hypercortisolism. In fact, it has recently been proposed that subclinical CS is the most common clinical presentation of PMAH (1). Most cases of the disease manifest during the fifth and sixth decades of life, a later age of onset compared with other causes of CS (1). Therefore, young asymptomatic carriers of germline *ARMC5* mutations were expected, such as patients V-2 and V-3. A mutation carrier was identified after the sixth decade of life (patient III-18), allowing the assumption that the disease might also show incomplete penetrance (Figure 1). The 3 unaffected female patients (V-2, V-3, and III-18), carrying a germline *ARMC5* mutation, were 29, 25, and 76 years old, respectively. They exhibited no clinical features or laboratory evidence of CS and had normal adrenal CT scans (Supplemental Table 2 and Figure 2). Another 3 female family members with a germline *ARMC5* mutation (patients IV-13, IV-20, and IV-24) showed discordance between the laboratory findings and the adrenal CT scans. Patient IV-13 exhibited laboratory evidence of CS and a normal adrenal CT scan, and patients IV-20 and IV-24 had no laboratory evidence of CS but abnormal adrenal CT scans (Supplemental Table 2). The Knudson's 2-hit model and the timing of somatic mutations might account for the late onset of the disease and may explain in part its incomplete penetrance. Additional somatic mutations in other genes, the activation of different signaling pathways, or even environmental factors may also possibly modulate adrenal

enlargement, cortisol secretion and disease progression. It has already been shown that large adrenal lesions in PMAH accumulate more genomic abnormalities with the consequent activation of oncogenic pathways (39). The progression of the disease is usually slow, occurring over a period of many years. In one series of patients, the diagnosis of PMAH was delayed by a mean of 7.8 years (ranging from 1–20 years) (40).

PMAH is generally regarded as a bilateral adrenal disorder; therefore, the term bilateral macronodular adrenal hyperplasia has sometimes been used to designate the disease (2, 3). In contrast, in the largest studied family, nearly 38% (6 of 16) of the patients with PMAH showed radiological abnormalities in only 1 adrenal gland (a thickened and/or nodular adrenal), including the oldest family member with the disease (Table 1 and Supplemental Table 2 and Figure 2). Based on these findings, we believe that PMAH is a more appropriate name for this challenging disorder.

In conclusion, the present study demonstrates that inherited autosomal dominant mutations in the *ARMC5* gene are a frequent cause of PMAH. Therefore, all patients with PMAH or suspected of having the disease should be screened for mutations in the *ARMC5* gene so that they can receive an earlier diagnosis and treatment. Once a germline mutation is confirmed, first-degree relatives should also undergo genetic screening and counseling.

Acknowledgments

We thank Johanna Sandoval from Prognomix Inc (Montreal, Quebec, Canada) and Marcelo Reis, MD, PhD, from Personal Genome Diagnostics Inc (Baltimore, MD) for excellent technical assistance.

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This work was supported by grants from the São Paulo Research Foundation (FAPESP 2010/12702-1 to M.C.B.V.F.) and the Brazilian fostering agency Coordination for the Improvement of Higher Education Personnel (to G.A.A.). Génome Québec supported the genome-wide linkage study platform at Centre Hospitalier de l'Université de Montréal research center.

Disclosure Summary: The authors have nothing to disclose.

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