Endocrine (2017) 55:959–968
DOI 10.1007/s12020-016-0956-z

ORIGINAL ARTICLE

A multicenter experience on the prevalence of ARMC5 mutations in patients with primary bilateral macronodular adrenal hyperplasia: from genetic characterization to clinical phenotype

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Received: 11 February 2016 / Accepted: 7 April 2016 / Published online: 19 April 2016

Abstract *ARMC5* mutations have recently been identified as a common genetic cause of primary bilateral macronodular adrenal hyperplasia (PBMAH). We aimed to assess the prevalence of *ARMC5* germline mutations and correlate genotype with phenotype in a large cohort of PBMAH patients. A multicenter study was performed, collecting patients from different endocrinology units in Italy. Seventy-one PBMAH patients were screened for small mutations and large rearrangements in the *ARMC5* gene: 53 were cortisol-secreting (two with a family history of adrenal hyperplasia) and 18 were non-secreting cases of PBMAH. Non-mutated and mutated patients' clinical phenotypes were compared and related to the type of mutation. A likely causative germline *ARMC5* mutation was only identified in cortisol-secreting PBMAH patients (one with a family history of adrenal hyperplasia and ten apparently sporadic cases). Screening in eight first-degree relatives of three index cases revealed four carriers of an *ARMC5* mutation. Evidence of a second hit at somatic level was identified in five nodules. Mutated patients had higher cortisol levels ($p = 0.062$), and more severe hypertension and diabetes ($p < 0.05$). Adrenal glands were significantly larger, with a multinodular phenotype, in the mutant group ($p < 0.01$). No correlation emerged between type of mutation and clinical parameters. *ARMC5* mutations are frequent in cortisol-secreting PBMAH and seem to be associated with a particular pattern of the adrenal masses. Their identification may have implications for the clinical care of PBMAH cases and their relatives.

Keywords ARMC5 · Primary bilateral macronodular adrenal hyperplasia · Cushing's syndrome · Genotype to phenotype correlation

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Electronic supplementary material The online version of this article (doi:10.1007/s12020-016-0956-z) contains supplementary material, which is available to authorized users.

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Introduction

Primary bilateral macronodular adrenal hyperplasia (PBMAH), previously known as AIMAH (ACTH-independent macronodular adrenal hyperplasia), is responsible for <2 % of all cases of endogenous Cushing's syndrome (CS) [1]. Patients with PBMAH may show overt, subclinical, or cyclic hypercortisolism developing slowly after several years. As recently demonstrated, cortisol secretion appears to be regulated in these cases by intra-adrenal corticotrophin produced by a subpopulation of steroidogenic cells [2]. Bilateral adrenal masses may be non-secreting, however, and are usually diagnosed incidentally.

Important mechanisms underlying the pathogenesis of PBMAH have been elucidated, showing that cortisol secretion may be regulated by the aberrant expression of eutopic and ectopic G-protein coupled receptors such as GIP, vasopressin, LH, epinephrine/norepinephrine, and others, leading to cAMP pathway activation and steroidogenesis [3, 4]. Intra-adrenal corticotrophin production also has an important role in regulating cortisol secretion, acting as a local amplifier of the action of aberrant receptors [2]. An association with rare variants of the phosphodiesterases (i.e., *PDE11A*, *PDE8B*), key enzymes regulating intracellular cAMP levels, has been found in patients with PBMAH, and they probably play a part in the disease by altering cAMP degradation [5–7]. Activating somatic mutations of the *PRKACA* encoding the catalytic subunit α of protein kinase A have been reported as a frequent cause of cortisol-secreting adrenal adenomas [8]. Germline duplication on a genomic region of chromosome 19p, which includes *PRKACA*, was also identified in 5 of 35 patients (4 kindred) with cortisol-secreting adrenal hyperplasia [9].

The recent identification of germline mutations in the Armadillo Repeat Containing-5 gene (*ARMC5*; gene locus at 16p11.2) in several series of PBMAH has enabled the genetic grounds for the disease to be clarified. These mutations were detected in tumors obtained from 33 patients with the apparently sporadic form [10], whose adrenal nodules carried *ARMC5* biallelic mutations (one germline and one somatic), supporting a tumor-suppressor role for the *ARMC5* gene in the adrenal gland. Germline mutations were subsequently confirmed in apparently sporadic [11] and familial cases [12, 13]. Correlating genotype and phenotype in a large cohort of unrelated PBMAH patients showed that index cases carrying the mutation exhibited a more severe hypercortisolism and larger adrenal glands [14].

In this study, we searched for *ARMC5* mutations in a large cohort of Italian patients with cortisol-secreting and non-secreting PBMAH. The clinical features of the patients with and without *ARMC5* variants were analyzed, and the available published cases were reviewed to seek a possible correlation between the type of mutation and the severity of the disease.

Subjects and methods

Patients

The study involved 53 Caucasian patients with cortisol-secreting PBMAH—51 apparently sporadic and two kindred (Families A and B; Fig. 1); some of these patients had undergone surgery. Thirty-four patients had overt (1 cyclic) and 19 had subclinical CS. Another 18 patients with non-secreting PBMAH were also investigated. The patients' clinical features are given in a supplementary Table 1 (ST1). All patients underwent adrenal computed tomography (CT) scans or magnetic resonance imaging (MRI), which showed bilateral and macronodular adrenal disease. The maximum diameter of each adrenal gland and the sum of the diameters of the two glands were recorded.

Cortisol hypersecretion was confirmed on the basis of high levels of 24-h urinary-free cortisol (UFC), late-night serum or salivary cortisol, and lack of cortisol suppression after 1 mg dexamethasone challenge (normal values <50 nM/l). The adrenal origin of any cortisol hypersecretion was established from suppressed adrenocorticotropic hormone (ACTH) levels and confirmed by a negative corticotropin-releasing hormone test in cases without ACTH suppression. Subclinical CS was considered in patients with no signs or symptoms of hypercortisolism based on plasma cortisol levels between 50 and 138 nmol/l after 1 mg dexamethasone, and at least one other abnormal test result (high UFC levels, or ACTH levels below the reference range, or absent circadian cortisol rhythm).

Adrenal masses were considered as non-secreting after excluding pheochromocytoma (with at least two normal urinary fractionated metanephrine assays), primary aldosteronism (based on a serum aldosterone/renin activity ratio <30 after appropriate wash-out of any interfering drugs), and androgen hypersecretion in patients with bilateral adrenal incidentalomas.

In a subgroup of 18 CS patients, provocative tests were performed as described elsewhere [3] to search for any aberrant receptor expression.

The study was approved by the local ethical committee and conducted in accordance with the Helsinki Declaration. Informed consent was obtained from all patients.

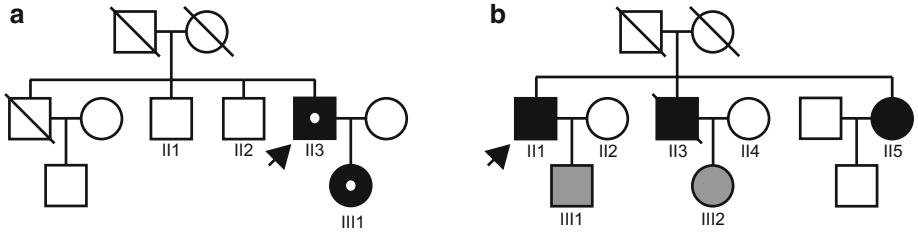

Fig. 1 Pedigree structures for PBMAH families (**a**, **b**). Only individuals assessed clinically and/or genetically have been numbered. Affected individuals met the classical clinical criteria for PBMAH. *Black-filled squares* and *circles* indicate affected males and females, respectively. *Gray-filled shapes* indicate cases with mild clinical signs. Probands are identified by *black arrows*. *Dots* inside a symbol represent an *ARMC5* mutation carrier

ARMC5 mutation analysis in lymphocyte and tumor DNA samples

Germline and tumoral DNA were isolated from peripheral blood and from snap-frozen adrenal nodules with the DNeasy Blood & Tissue Kit according to the manufacturer's protocol. *ARMC5* was analyzed in either lymphocyte DNA from 71 PBMAH patients (53 cortisol-secreting and 18 non-secreting) or tumoral DNA from 18 adrenal nodules from patients who underwent mono- or bilateral adrenalectomy. The whole coding region, the intron–exon boundaries, and the 5′- and 3′-UTRs were amplified using primers and conditions described elsewhere [10]. All the primer pairs used were synthesized by IDT (Leuven, Belgium). DNA sequencing was done with the BigDye 3.1 Termination Chemistry on an ABI 3730XL (Applied Biosystems, Monza, Italy).

Previously unreported nucleotide changes were sought in the dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP), ExAC browser (http://exac.broadinstitute.org), 1000 genomes (http://www.1000genomes.org), and the NHLBI Exome Sequencing Project—Exome Variant Server databases (http://evs.gs.washington.edu/EVS). Nucleotide changes described as common population-specific SNPs (minor allele frequency, MAF > 0.01) were disregarded. The SNP rs377719718, for which frequency data were not available in the public databases, was screened in 100 healthy, anonymous, unrelated individuals (see supplementary data). As variations between different alleles are caused by a difference in the number of repeat units (GGCCT), the PCR products generated using a *FAM*-labeled forward (5′-aggctgagcagaaggagtca-3′) and an unlabeled R primer (5′-tccaactccaaattgtttaatctca-3′) were separated on the ABI 3730XL DNA sequencer, and the data were analyzed with Peak Scanner v1.0 software (Applied Biosystems).

In *ARMC5* mutation carrier, for which snap-frozen tumor specimens were unavailable, the "second-hit" analysis was limited to the evaluation of loss of heterozygosity (LOH). In particular, sequence-based LOH analyses were performed, directly analyzing the *ARMC5* mutation on DNA extracted from 5-µm sections of archived paraffin-embedded tissues with the DNeasy Blood & Tissues kit (Qiagen) according to the manufacturer's instructions.

When available, patients' first-degree relatives were screened for *ARMC5* mutations by direct sequencing, as described above.

RNA extraction, reverse transcription, and RT-PCR

Total RNA was extracted from formalin-fixed and paraffin-embedded (FFPE) tissue using the miRNeasy FFPE kit (Qiagen) according to the manufacturer's recommendations. The RNA yield was determined on a nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Possible contamination of genomic DNA was removed by treating the total RNA with the Turbo DNA free kit (Applied Biosystems). Reverse transcription was done as reported elsewhere [15]. Briefly, 0.5 mg of DNase-treated RNA was reverse transcribed with random hexamers using the M-MuLV Reverse Transcriptase RNase H- (Euroclone, Pero, Italy) according to the manufacturer's recommendations. To test whether the c.438G>A and the c.174dupC mutation altered the splicing of *ARMC5* or induced mRNA decay, respectively, the surrounding cDNA sequence (exons 1–3) was amplified with specific primers (cARMC5_ex1F 5′-gctgtgtcgtcgtctagtcc-3′; cARMC5_ex3R 5′-gagggctaaggtcagtgcag-3′; cARMC5_ex1aF 5′-ctctgggtggggaaaaggac-3′; cARMC5_ex1R 5′-gtagcagcgcgagtaggg-3′).

Deletion analysis by qPCR

For those patients lacking informative biallelic polymorphism within the *ARMC5* gene sequence at germline level (i.e., hemi- and homozygous state could not be distinguished), the presence of possibly large rearrangements of the *ARMC5*-containing locus was assayed by qPCR. Adopting the salting out method, DNA was extracted from 300 µl of peripheral blood using the Gentra Puregene

Blood Kit (Qiagen) according to the manufacturer's instructions. The qPCR experiments were performed according to the MIQE guidelines [16]. Three different genomic regions of the *ARMC5*-containing locus (AC026471, Primer F1 5′-caacttccgacttgcatcac-3′, Primer R1 5′-tggaagacagggaaatcgtc-3′; Primer F2 5′-ctccgtaacctggcagactc-3′, Primer R2 5′-ggctaaggtcagtgcagcat-3′; Primer F3 5′-gacctggtgtctcccactgt-3′, Primer R3 5′-gccagcagaggttcataga-3′) were assessed with the GoTaq® qPCR Master Mix containing a carboxy-X-rhodamine passive reference dye (Promega, Milan, Italy) in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). A final concentration of 300 nM was used for both forward and reverse primers.

All samples were tested in duplicate on a MicroAmp 96-well reaction plate sealed with an optical adhesive film (Applied Biosystems) using 10 ng of DNA template in 20 µl of reaction mixture. No-template controls were included in each run. The qPCR conditions were 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 sec, and at 60 °C for 1 min. The data were analyzed with the Sequence Detection Software rel. 2.4 (Applied Biosystems), adopting an automatically set baseline and a fluorescence threshold adjusted for measuring Cq values. The amount of each target region relative to a reference locus on chromosome 17 (AC005703, Primer Fa 5′-aggtctgtgcgtgatgagtg-3′, Primer Ra 5′-tgtaggcgaaaccgtaggag-3′, final concentration of 300 nM for both primers) was ascertained using the ΔΔCq method, as the different regions showed comparable amplification efficiencies (100 ± 10 %).

Bioinformatic analysis

Four computational tools were used to predict the possible effects of the amino acid substitutions identified on protein function: PolyPhen-2, SIFT, MutationTaster, and MutPred. Only the following outputs were considered as deleterious variants: "probably or possibly damaging" for PolyPhen-2, "damaging" for SIFT, and "disease causing" for MutationTaster. The MutPred output comprises a general score (g) for the probability of the amino acid substitution being deleterious/disease associated, and scores for 5 top properties (p), where p is the *p* value indicating whether certain structural and functional properties are affected. A variant is only considered deleterious for combinations of *g* values >0.5 and a *p* value <0.05 for at least one property. Missense variants were considered damaging if they were termed as such by at least three of the four programs.

Data analysis and statistical methods

We calculated proportions and rates for categorical variables, and means ± standard deviations, or medians and inter-quartile ranges (IQR) for parametric or non-parametric variables. Groups were compared with the Chi-square test for categorical variables (or Fisher's exact test when the cell count was <5), or with the Mann–Whitney test for quantitative variables, as appropriate. The SPSS 17 software package (SPSS, Inc., Chicago, IL) was used to manage the dataset and for the statistical analyses. The level of significance was set at $p < 0.05$ for all tests.

Results

ARMC5 mutation analysis in PBMAH patients with CS

The patient cohort consisted of 53 PBMAH cases with CS (see Table S1). Genomic analysis of the whole *ARMC5* coding sequence and intronic boundaries led to the identification of 15 germline variants, one of which was in a familial case. Table 1 provides an overview of all the mutations identified, along with their intragenic locations, and predictive values of in silico analyses.

Two already-described nonsense substitutions [11, 14] were detected in our series. The first (c.1090 C>T, p.Arg364Ter) was found in a patient with sporadic disease (#3) and in two of her three children (Fig. 2a) who were studied for adrenal morphology and cortisol secretion. Evidence of a hyperplastic right adrenal gland with no evidence of cortisol hypersecretion was found in one of them (Subject II-1). The second mutation (c.2290 C>T, p.Arg764Ter) was identified in two sporadic cases, #40 and #41, in the Family A proband (#9), and—of the family members available for screening—only in his affected daughter (#8) (Fig. 1a).

Three frameshift mutations in exon 1 (c.174dupC, c.194delG, and c.220_222delinsTT) were detected in patients #19, #24, and # 47, respectively. These mutations are all novel and probably pathogenic (Table 1), leading to a downstream stop codon with premature termination of translation (p.Glu59Argfs44*, p.Gly65Alafs72*, p.Leu74Phefs63*, respectively), trimming out all *ARMC5* functional domains. Patient #24 shared the c.194delG with his son, but not with his sister, and no other first-degree relatives were available for genetic screening (Fig. 2b). Clinical details of the proband's son are not available.

Six patients harbored a missense substitution. Two are already known to be deleterious, i.e., the c.2192 C>G (p.Pro731Arg) and the c.1084 C>T (p.Arg362Trp), and were identified the former in patients #43 and #44, and the latter in patient #20. Another probably deleterious mutation involved a T to C transition (c.1739 T>C) causing the missense substitution p.Leu580Pro in exon 4, found here for the first time in patient #27 (see Table 1). Patient #28

Table 1 *ARMC5* nucleotide variants in PBMAH patients with overt or subclinical CS

DNA change (intragenic location)	Aminoacid change	SNP id (global MAF)[a] [allele frequency][b]	Mutation taster	PolyPhen-2	SIFT	MutPred[c]
c.-117 A>C	–	rs76210462	P	n/a	n/a	n/a
(Exon 1)		($C = 0.0054/27$)	(1.000)			
		[–]				
c.167G>C	p.Gly56Ala	–	P	B	T	0.317
(Exon 1)			(1.000)	(0.015)	(0.83)	
c.174dupC	p.Glu59Argfs44*	–	DC	n/a	D	n/a
(Exon 1)			(1.000)		(0.858)	
c.194delG	p.Gly65Alafs72*	–	DC	n/a	D	n/a
(Exon 1)			(1.000)		(0.858)	
c.220_222delinsTT	p.Leu74Phefs63*	–	DC	n/a	n/a	n/a
(Exon 1)			(1.000)			
c.325_326delinsT[d]	p.Pro109Serfs28*	–	DC	n/a	n/a	n/a
(Exon 1)			(1.000)			
c.438 G>A	p.Arg146Arg	rs201280100	DC	n/a	n/a	n/a
(Exon 1)		($A = 0.0038/19$)	(1.000)			
		[0.00828]				
c.1042delC[d]	L348Wfs27*	–	DC	n/a	n/a	n/a
(Exon 3)			(1.000)			
c.1084 C>T	p.Arg362Trp	–	DC	PD	D	0.673 (D)
(Exon 3)			(1.000)	(1.000)	(0.01)	
c.1090 C>T	p.Arg364Ter	–	DC	n/a	D	n/a
(Exon 3)			(1.000)		(n/a)	
c.1448 C>T	p.Pro483Leu	rs552657393	P	B	T	0.235
(Exon 4)		($G = 0.0006/3$)	(1.000)	(0.003)	(0.39)	
		[2.855e-05]				
c.1739 T>C	p.Leu580Pro	–	DC	PD	D	0.596 (D)
(Exon 4)			(1.000)	(1.000)	(0.01)	
c.1975 C>T[e]	p.Arg659Cys	(–)	P	B	D[f]	0.168
(Exon 4)		[4.187e−05]	(1.000)	(0.001)		
c.2192 C>G	p.Pro731Arg	rs200951744	DC	PD	D	0.186
(Exon 6)		($G = 0.0006/3$)	(0.992)	(0.859)	(0.03)	
		[0.001554]				
c.2290 C>T	p.Arg764Ter	–	DC	n/a	D	n/a
(Exon 6)			(1.000)		(n/a)	
c.*234_*238dup	–	rs377719718	P	n/a	n/a	n/a
(3′ UTR)		(–)	(1.000)			
		[–]				

Only SNPs with a minor allele frequency <1 % are reported. Unless stated otherwise, the nucleotide position refers to the RefSeq NM_001105247 and the variant identified at germline DNA level

DC disease causing, *P* polymorphism, *PD* probably damaging, *B* benign, *D* damaging, *T* tolerated, *n/a* not available

[a] Based on the 1000 Genome phase 1 population

[b] Based on the Exome Aggregation Consortium (ExAC)

[c] The reported general score (g) represents the probability of a deleterious mutation. A variant was considered damaging (D) only for g values >0.5 associated with p values <0.05 for alterations in certain structural and functional properties

[d] Found in tumoral DNA

[e] Position refers to the RefSeq NM_024742

[f] Low confidence

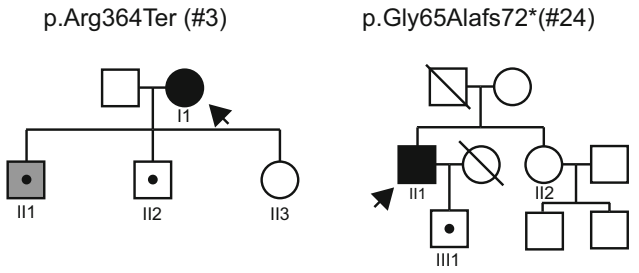

Fig. 2 Pedigrees of "sporadic" patients with *ARMC5* mutations. On the *left*: pedigree of patient #3 carrying the c.1090 C>T mutation; on the *right*: family tree of patient #24 carrying the small c.194delG deletion. Only individuals genetically screened for *ARMC5* mutations have been numbered. Affected individuals are represented by *black symbols*, while *gray-filled shapes* represent cases with mild clinical signs. Index cases are indicated by *arrows*, and *dots* inside a symbol indicate an *ARMC5* mutation carrier

revealed two different novel germline variants (c.167 G>C, p.Gly56Ala and c.1975 C>T, p.Arg659Cys), which most of the in silico models considered benign. Another novel missense substitution at exon 4 (c.1448 C>T, p.Pro483-Leu) was identified in patient #13 and was again judged to be benign and tolerated by three of the four computational methods (Table 1). Other heterozygous nucleotide changes with no apparent functional meaning were found (see supplementary data).

Germline DNA from 12 patients showing no clear signs of biallelic SNPs at the *ARMC5* locus, who were consequently potential carriers of a large genomic rearrangement, was assessed by qPCR, revealing no evidence of large deletions or duplications.

The whole *ARMC5* gene was then analyzed in tumoral DNA obtained from 18 nodules removed from 15 patients, regardless of any putative germline mutations (see ST1). Evidence of a tumoral second hit was only detected in tumor specimens from patients carrying an *ARMC5* causative germline mutation. In particular, evidence of LOH was detected in patients #3, #40, and #41, while two frameshift mutations—c.1042delC (p.L348Wfs27*) and c.325_326delinsT (p.Pro109Serfs28*)—were found in a second nodule from patients #40 and #24, respectively (Table 1). In all the remaining cases carrying rare alleles for SNPs rs201280100 (see supplementary data), including #11, there was no evidence of a tumoral second hit at the *ARMC5* locus. In patient #9, sequence-based LOH analysis on paraffin-embedded tissue slices was unsuccessful, suggesting the presence of a distinct, small somatic event in a different portion of the gene. This pattern was observed also in patient #19 in which, however, only the wild-type allele could be detected after tumor cDNA sequencing.

ARMC5 mutation analysis in non-secreting PBMAH

Eighteen non-secreting PBMAH patients were investigated. Apart from the non-synonymous change p.G930G (rs61734240) identified in two cases (#65, #57), no further significant nucleotide changes came to light, even after testing for large deletions/duplications in four patients with no evidence of biallelic SNPs after Sanger sequencing.

Correlating genotype with phenotype

Fifty-three patients with cortisol-secreting CS (12 mutated, including both affected subjects of Family A, and 41 wild-type cases) were considered for the genotype to phenotype correlation (Table 2). One patient in the initial cohort was excluded due to a lack of biochemical data. The age of the patients in the mutated and wild-type groups was similar, while there was a predominance of female patients in the latter group. There were more cases of overt CS, and higher UFC levels among the mutated patients, but the difference was not statistically significant ($p = 0.062$). Hypertension was more common and more severe in the mutated group, as demonstrated by the larger number of drugs needed to correct it ($p < 0.05$). HBA1c levels were higher in the mutated group too ($p = 0.041$), while no differences emerged between the two groups' BMI. Mutated patients had larger adrenal masses (for right plus left adrenals, $p < 0.01$), and a particular multinodular phenotype (see Supplementary Fig. 1). When patients with non-secreting PBMAH were included in the analysis, the difference in terms of adrenal size persisted, with mutated patients again having larger adrenal masses than the wild-type group (Table 3, $p < 0.001$).

Cortisol response to different provocative tests was analyzed in a subgroup of 18 PBMAH patients with CS: a positive response to upright posture was found in 9/14 patients (64 %), one carrying the $ARMC5^{R364X}$ (patient #3). Cortisol response to vasopressin/terlipressin stimulation was found in 4/13 patients (31 %), one of whom was an $ARMC5^{R764X}$ carrier (patient #8), while the mutated patient #19 showed a positive response to metoclopramide. None of the patients responding to the meal/OGTT or GnRH tests had mutations.

Cortisol levels were then analyzed in 6 cases (3 mutated and 3 wild-type patients) who underwent unilateral adrenalectomy based on adrenal asymmetry criteria (the largest gland on CT, and the predominant unilateral uptake on scintiscan). UFC levels returned to normal in the 3 mutated patients, remaining so at 12-month follow-up, and also in 2 of the 3 wild-type patients (see SF1).

We also sought to correlate phenotype severity with type of mutation in the light of a careful review of recently

Table 2 Clinical, biochemical characteristics, and adrenal morphology of 53 patients with cortisol-secreting PBMAH

Characteristics	Mutated ($n = 12$)	Non-mutated ($n = 41$)	p value
Age years	51.5 (36–62)	55.0 (28–78)	0.229
Sex (F/M)	6/6	36/5	0.005
CS/SCS	10/2	23/18	0.090
Hypertension	83 %	56 %	0.09
Number of anti-hypertensive drugs	2 (0–4)	1 (0–4)	0.013
BMI (kg/m^2)	28.5 (21–32)	27 (20–36)	0.560
UFC (times above ULN)[a]	2.2 (0.6–14.3)	1.2 (0.1–8.2)	0.062
Plasma cortisol after 1 mg dexamethasone (nmol/L)	341.0 (65–814)	168.0 (53–658)	0.234
ACTH < 10 ng/L (n)[b]	91.6	77.3	0.883
HbA1c (mmol/mol)	41.5 (27–129)	31 (25–56)	0.041
Size of adrenal gland			
Right (mm)	50.0 (21–72)	30.0 (10–85)	0.008
Left (mm)	56.0 (24–87)	30.0 (10–75)	0.001
Left + right (mm)	112.5 (24–145)	57.0 (24–128)	0.003

CS Cushing's syndrome, *SCS* subclinical Cushing's Syndrome

[a] UFC: urinary-free cortisol levels are expressed in times above the upper limit of normal (ULN)

[b] ACTH: normal values 10–50 ng/L

Table 3 Clinical, biochemical characteristics, and adrenal morphology of 71 PBMAH patients

Characteristics	Total ($n = 71$)	Mutated ($n = 12$)	Non-mutated ($n = 59$)	p value
Age (years)	56.5 (28–80)	51.5 (36–62)	59.0 (28–80)	0.049
Sex (F/M) %	69/31	66.7/33.3	69.5/30.5	0.847
Secreting/non-secreting %	53/18	100/0	57.7/25.3	0.027
Size of adrenal gland				
Right (mm)	30.0 (10–72)	50.0 (21–72)	26.0 (10–60)	0.001
Left (mm)	30.0 (10–100	58.5 (24–100)	27.0 (10–100)	<0.001
Left + right adrenal (mm)	100) 59.0 (20–188)	117.5 (24–188)	55.0 (20–150)	0.001

published series [10–14, 17], including all nonsense and frameshift mutations, and missense variants classified as pathogenic (see Fig. 3). When we scored the clinical features of our 53 mutated patients, we found no correlation between type of *ARMC5* mutation and level of hypercortisolism ($p = 0.787$), sex ($p = 0.527$), or age at onset ($p = 0.551$). Even when the size of the lesions was considered (in the 27 cases for which the data were comparable), no clear correlation with genotype emerged ($p = 0.561$).

Discussion

In this study, a molecular genetic analysis of *ARMC5* was performed in 71 Caucasian individuals (53 clinically diagnosed with cortisol-secreting and 18 non-secreting PBMAH), and the probands' genotype and phenotype were correlated. These cases form the second largest series of patients were genetically analyzed to be reported to date.

A likely causative germline *ARMC5* mutation was identified in all the individuals affected in one familial case and in ten apparently sporadic cases with cortisol-secreting PBMAH, indicating a prevalence similar to that of previous reports (11/53, 21 %) [11, 14]. No relevant nucleotide changes in the *ARMC5* gene were identified in the group of non-secreting PBMAH, which goes to show the marked heterogeneity of this disease. Likewise, no *ARMC5* germline mutations were detected in a recent study on patients with primary aldosteronism (PA) and detectable bilateral adrenal alterations [18]. On the other hand, another similar study on PA patients revealed damaging germline *ARMC5* variants in nearly 11 % of cases [19]—who were all African Americans, however, pointing to a role for ethnic background in this condition.

As in previous reports, our approach aimed not only at detecting punctual mutations by sequencing, but also at

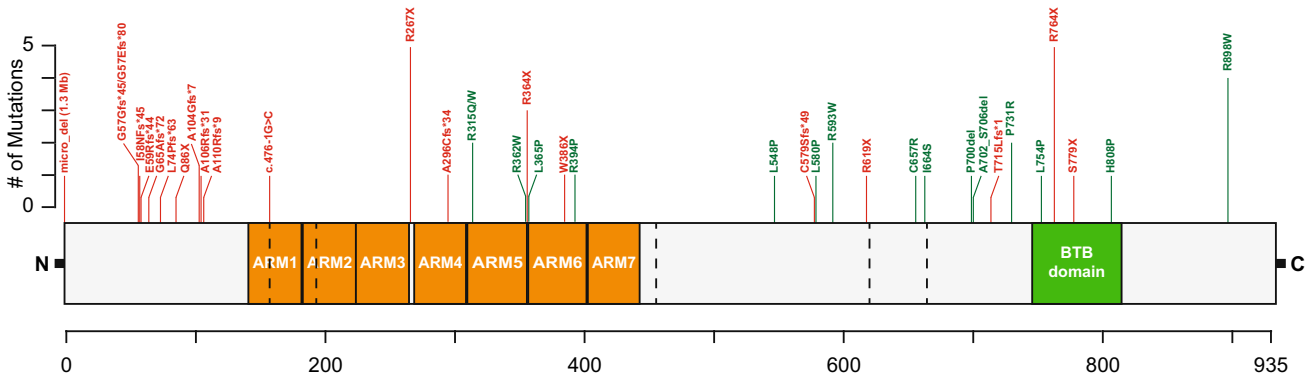

Fig. 3 Schematic representation of the ARMC5 gene (NM_001105247) with the germline mutations identified in this study or already reported [10–14, 17], which were used to seek a correlation between the severity of the phenotype and the type of ARMC5 mutation. Truncating mutations (nonsense and frameshift mutations and a large deletion) are shown in *red*, missense mutations—included in frame deletions—classified as pathogenic (see main text) in *green*. *Vertical dashed bars* identify exon boundaries. Protein domains are also shown in *orange* (Armadillo domain) and *green* (BTB/POZ domain) *boxes* (Color figure online)

large deletions or duplications by qPCR analysis. The consistent rate of mutation detection in the published studies suggests that large germline rearrangements in the *ARMC5* gene are rare—to our knowledge, only two cases have been described to date [10, 20]. In our cohort, all types of mutation (three of them novel) were found spread throughout the gene, without any hot-spots, and this is also consistent with previous studies, where most of the mutations observed were specific to each proband and only a minority of them recurred. Nonsense and frameshift mutations leading to nonsense transcripts accounted for nearly 60 % of all probably pathogenic variants found in our series. Although they might be translated, generating a protein with some missing functional domains, it is quite likely that most *ARMC5* nonsense transcripts are reduced by nonsense-mediated decay (which selectively eliminates mRNAs containing premature termination codons), an accepted mediator mechanism in the deleterious process of truncating mutations [21]. This hypothesis is supported by the fact that only the wild-type allele was detected in tumoral RNA deriving from the c.174dupC carrier.

According to previous published data, mutated patients with cortisol-secreting (overt and subclinical) PBMAH tended to have higher UFC levels, and more severe hypertension and diabetes, and highly significant differences emerged when we compared the size of the adrenal masses (even if we included patients with non-secreting PBMAH), mutated patients having larger adrenal lesions with a particular multi- and macronodular phenotype [11, 14].

Clinical diversity also emerged within the subgroup of mutated patients (see ST1): we hypothesized that this heterogeneity might correlate with the type of *ARMC5* mutation (i.e., missense or truncating), as seen in other single-gene disorders (e.g., pulmonary arterial hypertension [22] and X-linked dominant hypophosphatemic rickets [23]). This is not the case in PBMAH due to *ARMC5* mutations, however, since no correlation could be identified between the type of mutation and the available clinical parameters. This variability is therefore more likely to be influenced by other factors, including epigenetics and modifier genes, or by the wide array of second-hit mutations arising in the adrenal gland of PBMAH patients [24]. According to previous reports, in our series in the *ARMC5*-mutated group, males and females were equally distributed, whereas a marked female predominance was found in the unmutated [11, 14]. Although the reason of such discordance are not clear, we believe that in the sporadic cases, the pathogenesis might be more likely influenced by gender-related factors [25] than to inherited-genetic ones.

Although the function of the *ARMC5* gene remains largely unknown, in vitro analysis using the human adrenocortical cancer cell line H295R showed that transfecting the wild-type *ARMC5* gene-induced apoptosis, while introducing *ARMC5* missense mutations abolished this effect [10, 14]. A decrease in ARMC5 levels also prompted a reduction in ACTH receptor (MC2R) levels and a slight drop in the levels of two steroidogenic enzymes (CYP17A1 and CYP21A2), leading to a significant decrease in cortisol secretion [10]. This suggests that these patients' cortisol excess could be partly due to adrenal gland enlargement. In agreement with this hypothesis, although the number of cases is low—three patients carrying an *ARMC5* mutation—our data showed that a significant reduction of adrenal mass after unilateral adrenalectomy of the largest gland was associated with an abrupt drop in urinary cortisol levels at least in the short time. Analyzing a larger series with a longer follow-up might shed light on whether mutated patients would benefit

from unilateral adrenalectomy or warrant a more aggressive management [26, 27].

Illegitimate membrane receptor expression was tested in a subgroup of our patients: abnormal responses after upright posture and vasopressin/terlipressin administration were unassociated with the *ARMC5* status. Cortisol response to GnRH and OGTT/meal tests was only found in the wild-type patients, confirming the hypothesis that *ARMC5* mutations are associated with a particular spectrum of illicit receptors [10, 14].

In conclusion, identifying the involvement of *ARMC5* mutations in PBMAH represents a key advance in our understanding of the genetics of adrenal CS. Data emerging in our series further support this link, emphasizing the association between *ARMC5* mutations and a particular PBMAH phenotype with adrenal masses that are multi- and macronodular, and more severely cortisol secreting, with no food-dependent CS. Currently available data on the postoperative course of *ARMC5*-mutated patients are still too scarce to enable any conclusions to be drawn as to whether these patients would benefit from a more or less aggressive surgical management. It should be mandatory to analyze and follow up the members of index cases' families.

Acknowledgments The authors would like to thank the following people for all their help in patients' collection and characterization during this study: Dr Laura Trementino, Dr Paola Sartorato, Dr Mattia Barbot, Dr Marialuisa Zilio, Dr Margherita Rizzato, and Prof Franco Mantero.

Funding This work was funded by the Italian Ministry of University and Research (MIUR) through the FIRB project no. RBAP1153LS.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

References

1. A. Lacroix, ACTH-independent macronodular adrenal hyperplasia. Best Pract. Res. Clin. Endocrinol. Metab. **23**, 245–259 (2009)
2. E. Louiset, C. Duparc, J. Young, S. Renouf, M. Tetsi Nomigni, I. Boutelet, R. Libe, Z. Bram, L. Groussin, P. Caron, A. Tabarin, F. Grunenberger, S. Christin-Maitre, X. Bertagna, J.M. Kuhn, Y. Anouar, J. Bertherat, H. Lefebvre, Intraadrenal corticotropin in bilateral macronodular adrenal hyperplasia. N. Engl. J. Med. **369**, 2115–2125 (2013)
3. N.M. Albiger, G. Occhi, B. Mariniello, M. Iacobone, G. Favia, A. Fassina, D. Faggian, F. Mantero, C. Scaroni, Food-dependent Cushing's syndrome: from molecular characterization to therapeutical results. Eur. J. Endocrinol. **157**, 771–778 (2007)
4. N. El Ghorayeb, I. Bourdeau, A. Lacroix, Multiple aberrant hormone receptors in Cushing's syndrome. Eur. J. Endocrinol. **173**, M45–M60 (2015)
5. A. Horvath, S. Boikos, C. Giatzakis, A. Robinson-White, L. Groussin, K.J. Griffin, E. Stein, E. Levine, G. Delimpasi, H.P. Hsiao, M. Keil, S. Heyerdahl, L. Matyakhina, R. Libe, A. Fratticci, L.S. Kirschner, K. Cramer, R.C. Gaillard, X. Bertagna, J.A. Carney, J. Bertherat, I. Bossis, C.A. Stratakis, A genome-wide scan identifies mutations in the gene encoding phosphodiesterase 11A4 (PDE11A) in individuals with adrenocortical hyperplasia. Nat. Genet. **38**, 794–800 (2006)
6. D. Vezzosi, R. Libe, C. Baudry, M. Rizk-Rabin, A. Horvath, I. Levy, F. Rene-Corail, B. Ragazzon, C.A. Stratakis, G. Vandecasteele, J. Bertherat, Phosphodiesterase 11A (PDE11A) gene defects in patients with acth-independent macronodular adrenal hyperplasia (AIMAH): functional variants may contribute to genetic susceptibility of bilateral adrenal tumors. J. Clin. Endocrinol. Metab. **97**, E2063–E2069 (2012)
7. A. Rothenbuhler, A. Horvath, R. Libe, F.R. Faucz, A. Fratticci, M.L. Raffin Sanson, D. Vezzosi, M. Azevedo, I. Levy, M.Q. Almeida, M. Lodish, M. Nesterova, J. Bertherat, C.A. Stratakis, Identification of novel genetic variants in phosphodiesterase 8B (PDE8B), a cAMP-specific phosphodiesterase highly expressed in the adrenal cortex, in a cohort of patients with adrenal tumours. Clin. Endocrinol. (Oxf.) **77**, 195–199 (2012)
8. G. Di Dalmazi, C. Kisker, D. Calebiro, M. Mannelli, L. Canu, G. Arnaldi, M. Quinkler, N. Rayes, A. Tabarin, M. Laure Jullie, F. Mantero, B. Rubin, J. Waldmann, D.K. Bartsch, R. Pasquali, M. Lohse, B. Allolio, M. Fassnacht, F. Beuschlein, M. Reincke, Novel somatic mutations in the catalytic subunit of the protein kinase A as a cause of adrenal Cushing's syndrome: a European multicentric study. J. Clin. Endocrinol. Metab. **99**, E2093–E2100 (2014)
9. F. Beuschlein, M. Fassnacht, G. Assie, D. Calebiro, C.A. Stratakis, A. Osswald, C.L. Ronchi, T. Wieland, S. Sbiera, F.R. Faucz, K. Schaak, A. Schmittfull, T. Schwarzmayr, O. Barreau, D. Vezzosi, M. Rizk-Rabin, U. Zabel, E. Szarek, P. Salpea, A. Forlino, A. Vetro, O. Zuffardi, C. Kisker, S. Diener, T. Meitinger, M.J. Lohse, M. Reincke, J. Bertherat, T.M. Strom, B. Allolio, Constitutive activation of PKA catalytic subunit in adrenal Cushing's syndrome. N. Engl. J. Med. **370**, 1019–1028 (2014)
10. G. Assie, R. Libe, S. Espiard, M. Rizk-Rabin, A. Guimier, W. Luscap, O. Barreau, L. Lefevre, M. Sibony, L. Guignat, S. Rodriguez, K. Perlemoine, F. Rene-Corail, F. Letourneur, B. Trabulsi, A. Poussier, N. Chabbert-Buffet, F. Borson-Chazot, L. Groussin, X. Bertagna, C.A. Stratakis, B. Ragazzon, J. Bertherat, ARMC5 mutations in macronodular adrenal hyperplasia with Cushing's syndrome. N. Engl. J. Med. **369**, 2105–2114 (2013)
11. F.R. Faucz, M. Zilbermint, M.B. Lodish, E. Szarek, G. Trivellin, N. Sinaii, A. Berthon, R. Libe, G. Assie, S. Espiard, L. Drougat, B. Ragazzon, J. Bertherat, C.A. Stratakis, Macronodular adrenal hyperplasia due to mutations in an armadillo repeat containing 5 (ARMC5) gene: a clinical and genetic investigation. J. Clin. Endocrinol. Metab. **99**, E1113–E1119 (2014)
12. G.A. Alencar, A.M. Lerario, M.Y. Nishi, B.M. Mariani, M.Q. Almeida, J. Tremblay, P. Hamet, I. Bourdeau, M.C. Zerbini, M.A. Pereira, G.C. Gomes, S. Rocha Mde, J.L. Chambo, A. Lacroix, B.B. Mendonca, M.C. Fragoso, ARMC5 mutations are a frequent cause of primary macronodular adrenal hyperplasia. J. Clin. Endocrinol. Metab. **99**, E1501–E1509 (2014)

13. L. Gagliardi, A.W. Schreiber, C.N. Hahn, J. Feng, T. Cranston, H. Boon, C. Hotu, B.E. Oftedal, R. Cutfield, D.L. Adelson, W.J. Braund, R.D. Gordon, D.A. Rees, A.B. Grossman, D.J. Torpy, H.S. Scott, Armc5 mutations are common in familial bilateral macronodular adrenal hyperplasia. J. Clin. Endocrinol. Metab. (2014). doi:10.1210/jc.20141265
14. S. Espiard, L. Drougat, R. Libe, G. Assie, K. Perlemoine, L. Guignat, G. Barrande, F. Brucker-Davis, F. Doullay, S. Lopez, E. Sonnet, F. Torremocha, D. Pinsard, N. Chabbert-Buffet, M.L. Raffin-Sanson, L. Groussin, F. Borson-Chazot, J. Coste, X. Bertagna, C.A. Stratakis, F. Beuschlein, B. Ragazzon, J. Bertherat, armc5 mutations in a large cohort of primary macronodular adrenal hyperplasia: clinical and functional consequences. J. Clin. Endocrinol. Metab. **100**, E926–E935 (2015)
15. G. Occhi, M. Losa, N. Albiger, G. Trivellin, D. Regazzo, M. Scanarini, J.L. Monteserin-Garcia, B. Frohlich, S. Ferasin, M.R. Terreni, A. Fassina, L. Vitiello, G. Stalla, F. Mantero, C. Scaroni, The glucose-dependent insulinotropic polypeptide receptor is overexpressed amongst GNAS1 mutation-negative somatotropinomas and drives growth hormone (GH)-promoter activity in GH3 cells. J. Neuroendocrinol. **23**, 641–649 (2011)
16. S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. **55**, 611–622 (2009)
17. U. Elbelt, A. Trovato, M. Kloth, E. Gentz, R. Finke, J. Spranger, D. Galas, S. Weber, C. Wolf, K. Konig, W. Arlt, R. Buttner, P. May, B. Allolio, J.G. Schneider, Molecular and clinical evidence for an ARMC5 tumor syndrome: concurrent inactivating germline and somatic mutations are associated with both primary macronodular adrenal hyperplasia and meningioma. J. Clin. Endocrinol. Metab. **100**, E119–E128 (2015)
18. P. Mulatero, F. Schiavi, T.A. Williams, S. Monticone, G. Barbon, G. Opocher, F. Fallo: ARMC5 mutation analysis in patients with primary aldosteronism and bilateral adrenal lesions. J. Hum. Hypertens. doi:10.1038/jhh.2015.98 (2015)
19. M. Zilbermint, P. Xekouki, F.R. Faucz, A. Berthon, A. Gkourogianni, M.H. Schernthaner-Reiter, M. Batsis, N. Sinaii, M.M. Quezado, M. Merino, A. Hodes, S.B. Abraham, R. Libe, G. Assie, S. Espiard, L. Drougat, B. Ragazzon, A. Davis, S.Y. Gebreab, R. Neff, E. Kebebew, J. Bertherat, M.B. Lodish, C.A. Stratakis, Primary aldosteronism and ARMC5 variants. J. Clin. Endocrinol. Metab. **100**, E900–E909 (2015)
20. S. Suzuki, I. Tatsuno, E. Oohara, A. Nakayama, E. Komai, A. Shiga, T. Kono, T. Takiguchi, S. Higuchi, I. Sakuma, H. Nagano, N. Hashimoto, T. Mayama, H. Koide, H. Sasano, Y. Nakatani, T. Imamoto, T. Ichikawa, K. Yokote, T. Tanaka, Germline deletion of Armc5 in familial primary macronodular adrenal hyperplasia. Endocr. Pract. **21**, 1152–1160 (2015)
21. M. Khajavi, K. Inoue, J.R. Lupski, Nonsense-mediated mRNA decay modulates clinical outcome of genetic disease. Eur. J. Hum. Genet. **14**, 1074–1081 (2006)
22. E.D. Austin, J.A. Phillips, J.D. Cogan, R. Hamid, C. Yu, K.C. Stanton, C.A. Phillips, L.A. Wheeler, I.M. Robbins, J.H. Newman, J.E. Loyd, Truncating and missense BMPR2 mutations differentially affect the severity of heritable pulmonary arterial hypertension. Respir. Res. **10**, 87 (2009). doi:10.1186/1465-9921-10-87
23. M. Morey, L. Castro-Feijoo, J. Barreiro, P. Cabanas, M. Pombo, M. Gil, I. Bernabeu, J.M. Diaz-Grande, L. Rey-Cordo, G. Ariceta, I. Rica, J. Nieto, R. Vilalta, L. Martorell, J. Vila-Cots, F. Aleixandre, A. Fontalba, L. Soriano-Guillen, J.M. Garcia-Sagredo, S. Garcia-Minaur, B. Rodriguez, S. Juaristi, C. Garcia-Pardos, A. Martinez-Peinado, J.M. Millan, A. Medeira, O. Moldovan, A. Fernandez, L. Loidi, Genetic diagnosis of X-linked dominant Hypophosphatemic Rickets in a cohort study: tubular reabsorption of phosphate. BMC Med. Genet. (2011). doi:10.1186/1471-2350-12-116
24. R. Correa, M. Zilbermint, A. Berthon, S. Espiard, M. Batsis, G.Z. Papadakis, P. Xekouki, M.B. Lodish, J. Bertherat, F.R. Faucz, C.A. Stratakis, The ARMC5 gene shows extensive genetic variance in primary macronodular adrenocortical hyperplasia. Eur. J. Endocrinol. **173**, 435–440 (2015)
25. C.A. Stratakis, S.A. Boikos, Genetics of adrenal tumors associated with Cushing's syndrome: a new classification for bilateral adrenocortical hyperplasias. Nat. Clin. Pract. Endocrinol. Metab. **3**, 748–757 (2007)
26. N.M. Albiger, F. Ceccato, M. Zilio, M. Barbot, G. Occhi, S. Rizzati, A. Fassina, F. Mantero, M. Boscaro, M. Iacobone, C. Scaroni, An analysis of different therapeutic options in patients with Cushing's syndrome due to bilateral macronodular adrenal hyperplasia: a single-centre experience. Clin. Endocrinol. (Oxf) **82**, 808–815 (2015)
27. M. Iacobone, N. Albiger, C. Scaroni, F. Mantero, A. Fassina, G. Viel, M. Frego, G. Favia, The role of unilateral adrenalectomy in ACTH-independent macronodular adrenal hyperplasia (AIMAH). World J. Surg. **32**, 882–889 (2008)