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The *ARMC5* gene shows extensive genetic variance in primary macronodular adrenocortical hyperplasia

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Abstract

Objective—Primary macronodular adrenal hyperplasia (PMAH) is a rare type of Cushing's syndrome (CS) that results in increased cortisol production and bilateral enlargement of the adrenal glands. Recent work showed that the disease may be caused by germline and somatic mutations in the *ARMC5* gene, a likely tumor-suppressor gene (TSG). We investigated 20 different adrenal nodules from one patient with PMAH for *ARMC5* somatic sequence changes.

Design—All of the nodules where obtained from a single patient who underwent bilateral adrenalectomy. DNA was extracted by standard protocols and the *ARMC5* sequence was determined by the Sanger method.

Results—Sixteen of 20 adrenocortical nodules harbored, in addition to what appeared to be the germline mutation, a second somatic variant. The p.Trp476* sequence change was present in all 20 nodules, as well as in normal tissue from the adrenal capsule, identifying it as the germline defect; each of the 16 other variants were found in different nodules: 6 were frame shift, 4 were

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Declaration of interest

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

missense, 3 were nonsense, and 1 was a splice site variation. Allelic losses were confirmed in 2 of the nodules.

Conclusion—This is the most genetic variance of the *ARMC5* gene ever described in a single patient with PMAH: each of 16 adrenocortical nodules had a second new, “private”, and -in most cases- completely inactivating *ARMC5* defect, in addition to the germline mutation. The data support the notion that *ARMC5* is a TSG that needs a second, somatic hit, to mediate tumorigenesis leading to polyclonal nodularity; however, the driver of this extensive genetic variance of the second *ARMC5* allele in adrenocortical tissue in the context of a germline defect and PMAH remains a mystery.

Keywords

PMAH; Cushing’s syndrome; *ARMC5*; macronodular adrenal hyperplasia

Introduction

Primary Macronodular Adrenal Hyperplasia (PMAH) also known in the past as Bilateral Macronodular Adrenal Hyperplasia (BMAH) or ACTH-independent Macronodular Adrenal Hyperplasia (AIMAH) is a rare type of Cushing’s syndrome (CS) and is associated with bilateral enlargement of the adrenal glands. It accounts for less than 1% of all endogenous cases of CS[1]. The disease was first described by Kirschner *et al*. [2] in a single patient with ACTH-independent CS that developed over many years and was caused by apparently autonomously functioning multiple adrenal macronodules in both glands. In PMAH, there is an aberrant adrenal function of G-protein coupled receptors that can lead to cell proliferation and abnormal regulation of steroidogenesis[3]. Recently, Louiset *et al*. suggested that the hypercortisolism associated with PMAH appears to be corticotropin-dependent which challenged the notion of an ACTH independent disorder[4]. They found expression of proopiomelanocortin (POMC) messenger RNA in all samples of hyperplastic adrenal tissue and ACTH was detected in steroidogenic cells disseminated throughout the adrenal specimens. The release of adrenal ACTH was stimulated by ligands of aberrant membrane receptors but not by ACTH-releasing hormone or dexamethasone; in addition, an ACTH-receptor antagonist significantly inhibited in vitro cortisol secretion[4].

Although several patients have been described with mutations in various genes [5, 6], it was believed, that most cases of PMAH were sporadic. An autosomal dominant pattern of transmission was suggested for the familial cases [1, 5–7]. Most recently, Assie et al. found that the disease is caused by germline mutations in the in the armadillo repeat containing 5 (*ARMC5*) gene [8], in addition to somatic mutations in the tumor tissue; other studies also showed frequent *ARMC5* mutations in PMAH [7–9]. These findings confirmed previous data that suggested that the different nodules of PMAH represent products of polyclonal proliferative events that were propagated by changes in micro-RNAs, 17q22–24 losses and the involvement of multiple signaling pathways including those of cAMP, and Wnt[10–13]. *ARMC5* mutations were found in some of the tissues used in these studies, as well in previously described families with PMAH [14,15]. The *ARMC5* gene appears to function as a tumor-suppressor (TSG) and is located on chromosome 16 (16p11.2)[8]. However, little is

known about the way tumor form due to *ARMC5* loss, and more importantly nothing is known about what drives polyclonality in PMAH.

In the present investigation, we report a patient with PMAH caused by a germline *ARMC5* mutation who demonstrated extensive genetic diversity at the tissue level. To our knowledge, this phenomenon has not been described in other benign tumor disorders besides PMAH and is akin to what is seen in the context of malignancy-predisposing lesions, such as for example multiple colonic polyps of patients with hereditary predisposition to colon cancer[16].

Subjects and Methods

Clinical research protocol

The patient was admitted to the National Institute of Health (NIH) Warren Magnuson Clinical Center for the work-up and treatment of her PMAH under research protocol 00-CH-0160 (clinical trial registration number of NCT00005927). The *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Institutional Review Board approved this study, and informed consent was obtained from the patient.

Hormone assays

Plasma cortisol and ACTH levels, 24-hour (hr) urinary free cortisol (UFC) and 17-OH steroids were measured as described elsewhere[14].

ARMC5 sequencing analysis

DNA was obtained from 20 different adrenal nodules that were dissected from the surgically obtained specimen; the capsule of the adrenal gland was used for normal tissue. DNA was extracted according to manufacturer protocols (Qiagen, Valencia, CA, USA). *ARMC5* (OMIM: 615549; Chr16:31,470,317–31,478,488 – GRCh37/hg19) was analyzed in 20 different adrenal nodules and in one piece of normal tissue. The complete *ARMC5*-coding and surrounding intronic sequence, harboring all known isoforms, of these adrenal nodules and normal tissue was amplified using the conditions previously described[14]. Each PCR product was amplified using BigDye® Terminator V3.1 (Life Technologies, Grand Island, NY) purified using ZR DNA Sequencing Clean-up Kit™ (Zymo Research, Irvine, CA) and analyzed by classical bi-directional Sanger sequencing. For the variations nomenclature, the main frequent isoform in the literature (NM_001105247.1) was used.

In silico analysis & Immunohistochemistry

The *in silico* software tool “Polymorphism Phenotyping v2” (PolyPhen-2) was utilized to predict the pathogenic potential of the identified missense variants in *ARMC5*, as previously described[14]. *ARMC5* immunohistochemistry (IHC) was performed on tissues embedded in paraffin as previously described[14]. Unfortunately additional slides were not available in order to look for expression of *ARMC5* in the individual nodules corresponding to the somatic variants that were identified.

3D Computed tomography (CT) of adrenal glands

Surface rendering of the adrenal nodules was produced after precisely delineating them from CT scans in a semi-automated way. Afterwards, segmented adrenal surfaces were fused with the volumetric rendering of the abdomen region from CT images.

Results

Case presentation

A 42-year-old Caucasian female with no significant past medical history (including absence of meningioma and/or other tumors) presented to the NIH Clinical Center for evaluation of possible CS. Her medical history included secondary amenorrhea since the age of 38, a 12 kg weight gain over the previous two years, hirsutism, proximal muscle weakness, easy bruising and thinning of the skin. Family history was relevant for the presence of a meningioma in her father, but no other cancers or known CS. The biochemical evaluation revealed elevated 24-hr UFC (270 mcg/24 h, reference range 8–77 mcg/24 h), elevated late-night serum cortisol level (21.8 mcg/dl) and suppressed ACTH (<1 pg/ml, reference range 9–52 pg/ml). Serum cortisol remained unsuppressed after 1 mg overnight dexamethasone test (at 21.9 mcg/dl, normal <1.8 mcg/dl). CT imaging of the adrenal glands revealed bilateral multiple lobular masses more than 1 cm each in diameter, without evidence of cysts or microcalcifications (Figure 1 A, B). She was diagnosed with macronodular BAH. Glucagon-, GHRH-, mixed meal-, postural and vasopressin tests were performed in order to evaluate for aberrant hormonal responses (Table 1); the only positive one was the postural test. She underwent uncomplicated laparoscopic bilateral adrenalectomy. The largest nodule in the left side was 1.7 cm (Figure 1C) and in the right side was 2.5 cm (Figure 1D). Pathology was consistent with PMAH: multi-nodular glands with homogenous, golden-yellow-colored nodules, with no necrosis or hemorrhages. Nodules contained predominantly clear cells with interspersed compact cells disposed in nest and cord-like arrangement (Figure 2). The patient was discharged home in good condition on oral hydrocortisone and fludrocortisone replacement therapy and remains well to this day.

ARMC5 genetics and expression

We identified one *ARMC5* (NM_001105247.1) germline sequence variant that was present in all analyzed tissue and appeared to be the causative mutation; we also identified 14 somatic variants and two events consistent with losses of heterozygozity (LOH) in all 20 adrenal nodules (Figure 3 and Supplemental figure 1). The *ARMC5* nonsense variant c. 1428G>A (p.Trp476*) was present in all analyzed specimens, including tissue from the normal capsule. Other genetic defects were present in different nodules. Six of the variations were frameshift: c.327delC (p.Ala110Profs*27), c.346delT (p.Ser116Argfs*21), c.608delG (p.Ser203Thrfs*2), c.789_808del20 (p.Glu264Profs*5), c.1059_1080del22 (p.Cys353*), c. 2444delG p.Ala815Leufs*102); three were nonsense: c.807C>A (p.Cys269*), c.1033C>T (p.Gln345*), c.1059C>A (p.Cys353*); four were missense and resulted in amino acid substitutions: c. 247G>C (p.Ala83Pro), c.1751T>A (p.Val584Glu), c.2228C>T (p.Ala743Val), c.2405C>G (p.Pro802Arg); one was a splice site: c.476-1G>A; and in two nodules LOH was identified (Table 2). All sequence changes that were found in this patient

were novel. The supplemental figure 2 shows a 3D-CT with the lesions and the corresponding sequence variants found in each adrenal (left and right).

In silico analysis was performed for four somatic missense sequence variants and the prediction was that they were all likely damaging: p.Ala743Val, (score 0.703), p.Ala83Pro (score 1.000), p.Val584Glu (score 1.000), and p.Pro802Arg (score 1.000). It should be noted that scores vary from 0.000 to 1.000 and a greater score indicates a higher probability to impair ARMC5 protein function (Table 2).

IHC for ARMC5 was performed on our patient's samples and tissue from PMAH of a patient that did not have germline or somatic *ARMC5* variants (Figure 2). Cytoplasmic ARMC5 IHC was seen in the adrenal cortex of the patient with PMAH and no *ARMC5* sequence alterations, but was almost absent in our patient, confirming its loss at the protein level.

Discussion

In this study we identified 15 different *ARMC5* gene coding sequence alterations and two instances of LOH in a total of 20 analyzed nodules from the adrenals of a patient with PMAH. The *ARMC5* mutation p.Trp476* was present in all analyzed adrenal nodules and the normal tissue, making it the germline defect, or first "hit"; 14 of the nodules were found to have a second, nodule-specific somatic *ARMC5* defect. In another 2, there was LOH but in 4 there was neither LOH nor another variant. Although larger genomic losses or other rearrangements that would not be detected by the methods used in this study cannot be excluded in these remaining 4 nodules, the finding is consistent with those reporting *ARMC5* defects in patients with PMAH: not every nodule carried chromosome 16 LOH or another *ARMC5* variant [7–9]. The nodules where we did not identify a second mutation may either harbor a large deletion that cannot be identified by Sanger sequencing, mutation(s) in the middle of the intron changing the regular splicing of the RNA or, less likely, may reflect "contamination" of the studied tumor tissue with genetic material from normal cells. Another possibility is that somatic mutations in other gene(s) contribute to tumor formation at least as strongly as the germline *ARMC5* defect."

Does this mean that *ARMC5* haploinsufficiency alone can lead to adrenocortical proliferation? It is possible that this is the case and until this can be tested *in vitro* or in animal models we will not know for sure. However, it is clear that *ARMC5* haploinsufficiency leads to predisposition to developing these adrenocortical tumors. Could *ARMC5* deficiency lead to genomic instability as well? This is likely as the number of genetic variations and the degree of overall genomic diversity of nodules derived from the same patients with PMAH is extraordinary for what is, otherwise, a benign disorder. Only mutations associated with DNA and/or chromosomal instability are known to cause such diversity in pre-malignant conditions, the prime example of this being pre-malignant polyps in patients with *APC* or *MYH* mutations[17]. The issue of the need for bi-allelic, or whether monoallelic inactivation of genes like *MLH1*, *MSH2*, and *MYH* is sufficient to induce colonic tumorigenesis, is still under considerable debate in the literature; existing guidelines

recognize the association of specific phenotypes with single (dominant) and dual (recessive) losses, respectively[18].

What is remarkable in *ARMC5*'s mutiple and extenensive mutability is that PMAH is a benign disorder with no known cases of this disease ever progressing to adrenocortical cancer. Preliminary studies showed a possible TSG function for *ARMC5* as a protein that induces apoptosis (of the H295R cancer cell line)[8,19]. Thus, *ARMC5* inactivation leads to resistance to apoptosis in adrenocortical cells, which apparently leads to hyperplasia. However, the absence of malignancy also suggests that *ARMC5* inactivation does not cause a metastatic, aggressive cellular phenotype.

In conclusion, in this case study, we document extensive genetic variance of *ARMC5* in a single patient with PMAH. This adds to the existing body of evidence of extreme mutability of the *ARMC5* gene whose function remains to be determined in animal models and in in vitro studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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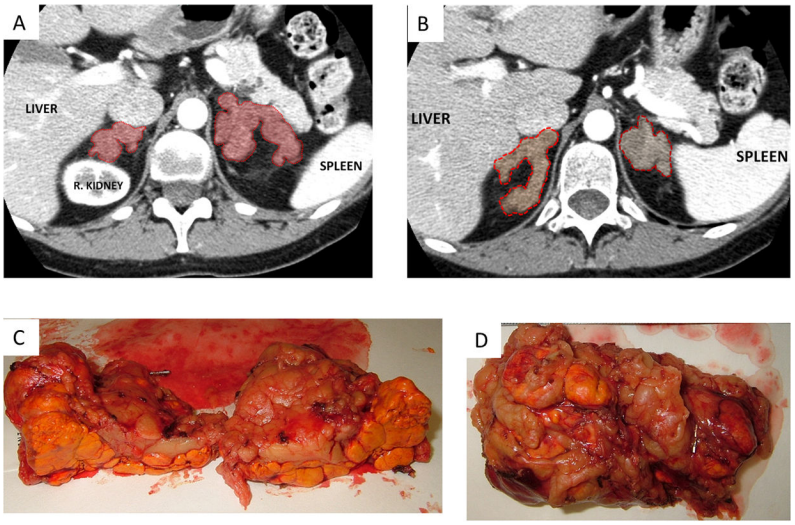

Figure 1.

Computed tomography of adrenal glands with volumetric rendering of the abdomen region (A,B). Gross pathology of the left adrenal gland (C). Gross pathology of the right adrenal gland (D).

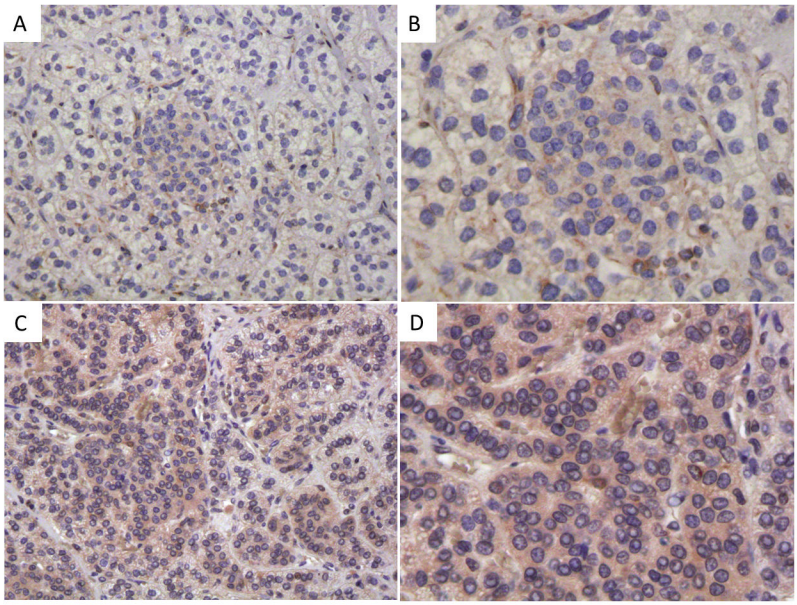

Figure 2.

ARMC5 immunohistochemistry: staining with an ARMC5-specific antibody was performed on a sample from the case presented here (A, B) and on tissues from a patient with PMAH and no *ARMC5* germline or somatic variants (C, D).

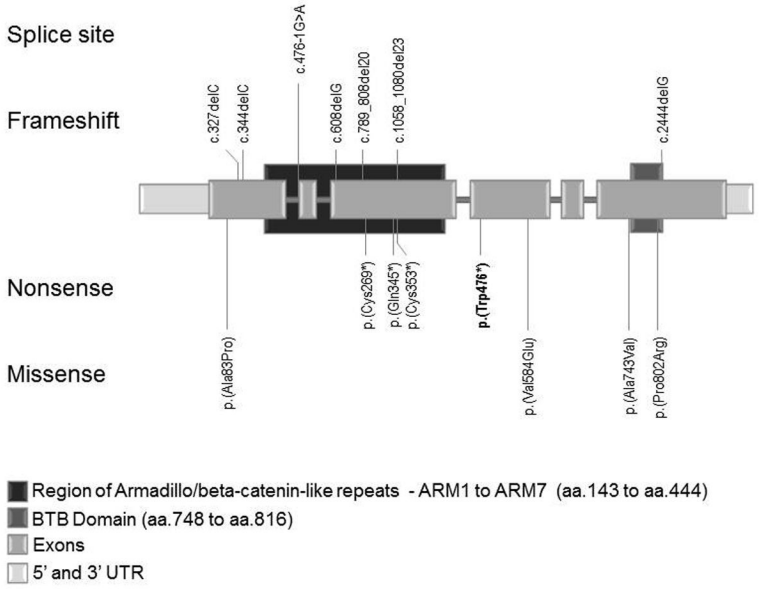

Figure 3.

Schematic representation of the *ARMC5* gene showing all variants identified in this report and their relative position in the gene and function in the protein; the germline mutation is shown in bold. The vertical lines show the variant position.

Abbreviations: aa=amino acids; UTR=untranslated region.

Table 1

different test to evaluate aberrant hormone receptor

Test	Variable	Time					
		minus 15 min	0 min	30 min	60 min	90 min	120 min
Postural Test*	Cortisol (mcg/dl)	17.4	17.8	23.5	25.8	27.4	29.8
	Aldosterone (ng/dl)	<1.5	<1.5	3.4	10.4	11.3	10.9
	Renin (ng/ml/hr)		<0.6		0.8		1
Mixed Meal test**	Aldosterone (ng/dl)		1.7	<1.5	<1.5	<1.5	<1.5
	Cortisol (mcg/dl)		20.8	20.2	16.2	17	16
GHRH test	Cortisol (mcg/dl)		19.8	22.1	22.9	22.9	21.7
	Aldosterone (ng/dl)		<1.5	1.6	2.6	2.6	1.5
	Growth Hormone ng/ml		1.4	2.7	1.8	0.8	0.4
Glucagon test	Cortisol (mcg/dl)	22	24	23.2	20.7	21.2	21.4
	Aldosterone (ng/dl)	<1.5	1.8	2.1	2.2	2.4	2.2
Vasopressin test	Aldosterone (ng/dl)		1.7	3.1	5.6	5.8	
	Cortisol (mcg/dl)		17.4	30.1	32.2	24.5	

* postural test positive

** mixed meal test negative

Table 2

Mutations found in the patient and the *in silico* modeling prediction of the effect of *ARMC5* missense substitution on the protein function

DNA change	Protein change	Exon	Domains	*In silico* modeling		Inter-species alignment			
				Prediction	Score[a]	*Mus musculus*	*Dasypus novemcinctus*	*Xenopus tropicalis*	*Petromyzon marinus*
c.247G>C	p.(Ala83Pro)	1	NTD	Damaging	1.000	A	-	A	A
c.327delC	p.(Ala110Profs*27)	1	NTD	Frameshift					
c.346delT	p.(Ser116Argfs*21)	1	NTD	Frameshift					
c.476-1G>A	splice	intron 1	Armadillo	Splice site					
c.608delG	p.(Ser203Thrfs*2)	3	Armadillo	Frameshift					
c.789_808del20	p.(Glu264Profs*5)	3	Armadillo	Frameshift					
c.807C>A	p.(Cys269*)	3	Armadillo	Nonsense					
c.1033C>T	p.(Gln345*)	3	Armadillo	Nonsense					
c.1059C>A	p.(Cys353*)	3	Armadillo	Nonsense					
c.1059_1080del22	p.(Cys353*)	3	Armadillo	Frameshift					
c.1428G>A	**p.(Trp476*)**	**4**	-	**Nonsense**					
c.1751T>A	p.(Val584Glu)	4	-	Damaging	1.000	V	V	I	V
c.2228C>T	p.(Ala743Val)	6	BTB/POZ-like	Damaging	0.703	A	A	-	-
c.2405C>G	p.(Pro802Arg)	6	BTB/POZ-like	Damaging	1.000	P	P	P	L
c.2444delG	p.(Ala815Leufs*102)	6	BTB/POZ-like	Frameshift					

[a]Scores goes from 0.000 to 1.000. Greater score indicates higher probability to impair the protein function. The main factors taken into account for the calculation of the score are: 1) difference in the thermo-physical properties of the wild type and mutant protein, and; 2) evolutionary preservation of the residue in the corresponding position.

NTD: n-terminal domain; BTB/POZ-like: BR-C, ttk and bab/pox virus and zinc finger like domain. The letters in the topic “Interspecies Alignment” are relative to the amino acid present in the position: V, Valine; I, Isoleucine; A, Alanine; P, Proline; L, Leucine.

“-“, no aminoacid present. In bold is the germline mutation.