ARMC5 mutations in a large French-Canadian family with cortisol-secreting β -adrenergic/vasopressin responsive bilateral macronodular adrenal hyperplasia

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Abstract

Background: Bilateral macronodular adrenal hyperplasia (BMAH) is a rare cause of Cushing's syndrome (CS) and its familial clustering has been described previously. Recent studies identified that *ARMC5* mutations occur frequently in BMAH, but the relation between *ARMC5* mutation and the expression of aberrant G-protein-coupled receptor has not been examined in detail yet.

Methods: We studied a large French-Canadian family with BMAH and sub-clinical or overt CS. Screening was performed using the 1-mg dexamethasone suppression test (DST) in 28 family members. Screening for aberrant regulation of cortisol by various hormone receptors were examined *in vivo* in nine individuals. Sequencing of the coding regions of *ARMC5* gene was carried out.

Results: Morning ambulating cortisol post 1 mg DST were > 50 nmol/l in 5/8 members in generation II (57–68 years old), 9/22 in generation III (26–46 years old). Adrenal size was enlarged at different degrees. All affected patients increased cortisol following upright posture, insulin-induced hypoglycemia and/or isoproterenol infusion. β -blockers led to the reduction of cortisol secretion in all patients with the exception of two who had adrenalectomies because of β -blockers intolerance. We identified a heterozygous germline variant in the *ARMC5* gene c.327_328insC, (p.Ala110Argfs*9) in nine individuals with clinical or subclinical CS, in four out of six individuals with abnormal suppression to dexamethasone at initial investigation and one out of six individuals with current normal clinical screening tests.

Conclusions: Systematic screening of members of the same family with hereditary BMAH allows the diagnosis of unsuspected subclinical CS associated with early BMAH. The relation between the causative *ARMC5* mutation and the reproducible pattern of aberrant β -adrenergic and V₁-vasopressin receptors identified in this family remains to be elucidated.

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Introduction

Primary adrenal etiologies of Cushing's syndrome (CS) are responsible for 15–25% of cases of endogenous overt hypercortisolism, which are mainly due to adrenal adenomas and carcinomas (1). Rarely, CS is secondary to primary bilateral adrenal hyperplasias (<2%), and this includes primary bilateral macronodular adrenal hyperplasia (BMAH) (2). Most BMAH cases were initially reported as sporadic, but more than ten kindreds of familial BMAH were reported in recent years, and in all cases their presentation suggested an autosomal dominant mode of transmission (3, 4, 5, 6, 7, 8, 9, 10). The true prevalence of hereditary BMAH may have been underestimated because familial screening was not performed systematically (2).

In the last two decades, the regulation of steroidogenesis in BMAH was elucidated in part following the identification of aberrant expression and function of several G-protein-coupled receptors (GPCR) in BMAH tissues (11). The aberrant stimulation of steroidogenesis can be under the control of ectopic receptors such as those for glucose-dependent insulinotropic peptide, catecholamines (β -adrenergic receptor), vasopressin (V_2 – V_3 -vasopressin receptor), serotonin (5-HT₇ receptor), glucagon, and probably angiotensin II receptor (AT1R). It can also result from increased expression or altered activity of eutopic receptors such as those for vasopressin (V_1 -vasopressin receptor), luteinizing hormone (LH)/human chorionic gonadotropin, serotonin (5-HT₄ receptor), and leptin receptor (11, 12). In addition, adrenocorticotrophic hormone (ACTH) can be produced in clusters of steroidogenic cells in BMAH tissues and regulate steroidogenesis in a paracrine manner (13). ACTH was not regulated by corticotropin-releasing hormone (CRH) or glucocorticoid receptor, but was stimulated by aberrant receptor activation. Cortisol production in BMAH is therefore modified both by aberrant GPCR and autocrine ACTH, which amplifies the aberrant receptor effects (13).

The genetic basis of hereditary BMAH appears to be heterogeneous (12, 14, 15). A limited number of cases of BMAH were described in association with the multiple endocrine neoplasia type 1 syndrome (14, 15) and the hereditary leiomyomatosis and renal cancer syndrome (14, 16). Although BMAH may be associated with McCune–Albright syndrome (*GNAS1*), only a few cases of sporadic BMAH with somatic *GNAS1* mutations were reported (16, 17). BMAH was also found in patients with familial colon polyps and adenomatous polyposis coli (*APC*) gene mutation (14, 16).

Recently, using single-nucleotide polymorphism (SNP) arrays, linkage analysis and whole genome sequencing, germline and somatic *ARMC5* gene mutations were identified in 18/33 (55%) patients with apparently sporadic BMAH and CS (18). Similarly, pathogenic *ARMC5* mutations were found in a second cohort of 34 apparently sporadic cases of BMAH with CS with a prevalence of 20.6% (19) and very recently *ARMC5*-damaging mutations were identified in 24/98 (26%) index cases of BMAH (20). Furthermore, we and others identified causal germline *ARMC5* mutations in six families with BMAH (21, 22, 23).

The relation between *ARMC5* mutation and the expression of aberrant GPCR in BMAH has not yet been examined in detail. Preliminary studies have found that *ARMC5* mutation carriers may present aberrant response to upright posture, vasopressin and metoclopramide tests; in contrast, none of the patients with food-dependent CS carried *ARMC5* mutations (18, 20, 23).

We describe a French-Canadian BMAH kindred with β -adrenergic and V_1 -vasopressin regulation of cortisol in which nine members of the family are affected, including seven with sub-clinical and two with clinical CS. A heterozygous germline *ARMC5* mutation was identified in the index case that segregates with the disease in the family.

Subjects and methods

Clinical details and endocrine investigations of probands

Proband II-1 ► We previously reported on this 56-year-old man with BMAH and CS with aberrant β -adrenergic and V_1 -vasopressin response (24) (Table 1 for endocrine investigation and radiological findings). In this patient, cortisol secretion increased during the change from supine to upright posture, the arginine–vasopressin test (10 IU i.m.), a treadmill stress test, an insulin-induced hypoglycemia and isoproterenol infusion (Figs 1 and 2) (24). Cortisol secretion was not modified by mixed meal, thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), glucagon, and cisapride. Treatment with the β -adrenergic-antagonist propranolol blocked the cortisol increase when upright. Since propranolol (up to 320 mg daily) greatly reduced but not normalized 24-h urinary free cortisol (UFC) levels, a left unilateral adrenalectomy was performed and the diagnosis of BMAH was confirmed at histology. Therapy with propranolol was resumed after the surgery and UFC

Table 1 Summary of clinical data and endocrine investigation of the nine members of the family affected by bilateral macronodular adrenal hyperplasia (BMAH).

	Patients									
	II-1	II-3	II-7	II-8	II-9	III-2	III-4	III-5	III-10	
Age (years)	56	65	59	59	56	46	44	42	44	
Sex (F, M)	M	F	M	F	F	M	F	F	M	
Clinical presentation	Phlebitis, weight gain	Familial screening	Adrenal incidentalomas	Familial screening	Familial screening	Familial screening	Familial screening	Familial screening	Familial screening	
Plasma cortisol after 1 mg dexamethasone (nmol/l) ^a	499 (2 mg)	333	489	98	580	85	650	251	119	
Urinary free cortisol (n: 90–330 nmol/day)	1963–2953	123	187–252	152	144	126	772	144	250	
Basal ACTH (n: 2–11 pmol/l)	0.86	1.6	1.3	4.3	<2.2	1.1	<0.5	<0.5	5.2	
4 mg-DEX IV (nmol/l) ^b	709	323	801	59	463	138	615	130	44	
Posture test	469–828 (177%)	325–940 (289%)	461–710 (154%)	46–128 (278%)	164–262 (160%)	47–89 (189%)	601–867 (144%)	129–216 (167%)	45–574 (1275%)	
Insulin-induced hypoglycemia test	709–1396 (197%)	ND	462–703 (152%)	27–131 (476%)	194–417 (215%)	39–232 (595%)	776–1151 (148%)	139–366 (263%)	ND	
Treadmill stress test	519–811 (156%)	609–664 (109%)	514–752 (146%)	75–81 (108%)	236–376 (159%)	ND	ND	ND	105–140 (133%)	
Isoproterenol	703–1446 (206%)	ND	416–937 (225%)	41–147 (359%)	170–478 (281%)	73–239 (327%)	438–944 (216%)	130–292 (225%)	28–148 (529%)	
Vasopressin	491–1057 (215%)	184–654 (355%)	715–998 (140%)	117–202 (173%)	216–474 (220%)	105–283 (269%)	458–895 (195%)	141–332 (235%)	82–162 (198%)	
Desmopressin	574–588 (102%)	163–117 (72%)	357–382 (107%)	54–66 (122%)	220–181 (82%)	ND	ND	ND	ND	
Right adrenal gland	Nodular 52×60 mm	One nodule 32 mm	Multiple nodules One nodule 19×15 mm (HU18) and one nodule 29×19 mm (HU25)	Nodule 9 mm	Two nodules of 8 mm	Nodule 8 mm	Nodular 30×20 mm	Nodular and one nodule 8.5 mm	Nodule 10 mm (HU24) and nodule 6 mm (HU14)	
Left adrenal gland	Nodular 65×85 mm	Nodular 35 mm	Multiple nodules 59×32 mm (HU24) and one nodule 30 mm	Nodule 7 mm	One nodule 13×8 mm and one nodule 23×20 mm (HU <10)	Nodule 25×16 mm (HU26)	Nodular 23×25 mm	Three nodules between 10 and 12 mm	Nodule 10 mm (HU15) and nodule 20 mm (HU26)	

ND, not done; F, female; M, male.

^an cortisol <50 nmol/l.^bn cortisol <76 nmol/l at 0900 h the morning following 4 mg-IV dexamethasone perfusion.

Figure 1

Endocrine investigation of individuals II-1 and II-7. We found that cortisol secretion in both subjects was stimulated by upright posture (circles) and the insulin tolerance test (triangles). The implication of β_1 -adrenergic receptor was confirmed by the stimulation of cortisol secretion by isoproterenol (squares).

became normalized with decreasing doses of propranolol to 20–40 mg daily. Seventeen months after the surgery, UFC increased up to 451 nmol/day (normal: 90–330) when propranolol was stopped inadvertently. Fifteen years following the initial adrenalectomy, abdominal computed tomography showed no change in the size of the right adrenal gland (Fig. 3). His UFC remain well controlled with atenolol 50 mg daily, while control of blood pressure required the addition of irbesartan–hydrochlorothiazide 300/12.5 mg, and nifedipine XL 20 mg daily. A posture test was repeated under atenolol

and showed an increase of plasma cortisol from 430 to 642 nmol/l (149%), which is slightly less than the initial increase of 177%. The most recent morning plasma ACTH value was low at <0.5 pmol/l (n : 2–11), while his UFC remained normal at 190 nmol/day (90–330).

Proband II-7 ▶ A 59-year-old brother of the first patient was referred 10 years later after his brother for an incidentally found bilateral adrenal enlargement (Table 1 and Fig. 2). He had been diagnosed with high blood pressure, requiring four medications and dyslipidemia since age 40. The patient reported asthenia, a weight gain of 36 kg during the last 4 years, and proximal muscle weakness during the last 2 years. Physical examination revealed high blood pressure (158/80 mmHg), facial plethora, and truncal obesity. Despite normal UFC levels, ACTH levels were low at 1.1 pmol/l with no increase following CRH stimulation test, and cortisol was not suppressed by overnight dexamethasone test (Table 1). Similarly to his brother, cortisol secretion was stimulated by upright posture (154%), a treadmill stress test (146%), insulin-induced hypoglycemia (152%), and isoproterenol infusion (225%) in addition to arginine–vasopressin (140%) (Table 1 and Fig. 1). Treatment with propranolol 120 mg twice daily did not completely block the increase in cortisol during upright posture (plasma cortisol from 174 to 334 nmol/l (192%)). The patient was treated with nadolol 20 mg twice daily for 10 months and the patient reported a weight loss of 7 kg, reduction in central obesity, and normalization of his hypertension. The posture test was repeated 10 months later under nadolol (normal UFC) and plasma cortisol increased from 314 to 486 nmol/l (154.8%). Vasopressin stimulation increased plasma cortisol from 312 to 624 nmol/l (200%), while the computed tomography (CT) scan showed no change in the adrenal glands size. Unfortunately, the patient died from a massive stroke 1 year later.

Clinical screening for prevalence of familial BMAH

First-degree adult family members of probands II-1 and II-7 were offered clinical screening for BMAH. All patients signed an informed consent under the research protocol approved by the Institutional Review Board of the Centre Hospitalier de l'Université de Montréal (CHUM).

Basal investigations included a complete medical evaluation, physical examination and a 1 mg overnight dexamethasone suppression test (DST) using a normal plasma cortisol cut-off <50 nmol/l. When 1 mg DST

Figure 2

Pedigree of a French-Canadian family with hereditary BMAH caused by the *ARMC5* c.327_328insC; p.Ala110Argfs*9 mutation. Circles indicate females; squares indicate males;

DST, dexamethasone suppression test; CS, Cushing's syndrome; +, *ARMC5* mutation present; -, *ARMC5* mutation absent.

showed abnormal suppression, a 24-h urine collection for measurement of UFC, 4 mg-IV DST (normal: plasma cortisol <76 nmol/l at 0900 h on the morning following dexamethasone) and a CT scan of adrenal glands with no contrast were performed.

The diagnostic criteria used for primary CS were: increased UFC (>330 nmol/day), morning plasma cortisol >50 nmol/l following overnight 1 mg DST, >76 nmol/l the morning following 4 mg-IV DST, and plasma ACTH levels <2 pmol/l (*n*: 2–11). The diagnostic criteria for sub-clinical CS included normal UFC with sub-normal suppression of plasma cortisol levels following 4 mg-IV DST (>76 nmol/l) and low basal plasma ACTH levels (<2 pmol/l) (25).

***In vivo* screening for the presence of aberrant hormone receptors**

In all patients with confirmed abnormal suppression to 4 mg-IV DST, the *in vivo* screening for the presence of

aberrant hormone receptors was performed as described previously (25, 26). In addition, in patients from generation II with normal suppression to 1 and/or 4 mg-IV DST, posture and vasopressin stimulations were performed as additional tests. Subjects with sub-clinical CS received dexamethasone 1 mg orally every 6 h for 24–48 h before and during all the investigations to ensure that ACTH levels remained suppressed.

Plasma levels of cortisol, ACTH, aldosterone, and renin were measured at 30–60 min intervals for 2–3 h during tests that transiently modulate the levels of ligands for potential aberrant receptors (11). All tests were done at fasting with the patient in supine posture for at least 60 min prior to the tests. On day 1, an upright posture test lasting 2 h was followed by a standard mixed meal, and by 1–24 ACTH, 250 µg i.v. (Cortrosyn, Organon Canada, Scarborough, ON, Canada). On a second day, the administration of 100 µg GnRH i.v. (Factrel, Wyeth-Ayerst, Montréal, QC, Canada) was followed by 200 µg TRH i.v. (Relefact, Hoechst-Roussel, Montréal, QC,

Figure 3

Adrenal CT scan of individuals II-3 and III-4 in the upper panel: they were both prospectively diagnosed with BMAH as they presented bilateral enlargement of their adrenal glands with multiple nodules. Adrenal CT scans of individual II-1 in 1995 and in 2007 showing no increase in size in the right adrenal gland after 13 years of therapy with β -blockers (lower panel).

Canada). Stimulation by 6 mg tegaserod orally (Zelnorm, Novartis Pharma Canada) or metoclopramide 10 mg and 10 IU arginine vasopressin im (Pitressin, Parke-Davis, Scarborough, ON, Canada) were performed on day 3. A vasopressin test was performed in some patients during intravenous infusion of 4 mg dexamethasone (1 mg/h starting 3 h before vasopressin injection).

The results of the tests were expressed as a percentage using the following calculation: Peak in cortisol/basal cortisol value at time 0 min $\times$ 100. A change of plasma cortisol levels of <125% was arbitrarily defined as no response, a 125–149% change as a partial response, and a change $\geq$ 150% as a positive response.

Confirmation of the catecholamine-responsive cortisol secretion

Further confirmation for the catecholamine-dependent cortisol secretion included the response to a treadmill stress test, an insulin 0.12 units/kg i.v. tolerance test and isoproterenol infusion at a rate of 20 ng/kg of body weight per min for 30 min with cortisol/ACTH/aldosterone and renin measured at –120, –60, –15, 0, 10, 20, 30, 45, 60, 75, 90, and 120 min.

Confirmation of the V₁-vasopressin-responsive cortisol secretion

When a significant response to vasopressin was found, further tests included a 2.5 μ g s.c. of V₂-vasopressin receptor agonist desmopressin (dDAVP, Ferring, Inc.).

Assays

Plasma cortisol, FSH, LH, thyroid-stimulating hormone, and prolactin were measured by immunofluorometric assay (Bayer Immuno I System, Tarrytown, NY, USA) and ACTH by immunoradiometric assay (Allegro, Nichols Diagnostics, San Juan Capistrano, CA, USA). Plasma aldosterone and renin activity were measured by RIA Kits (DSL, Webster, TX, USA).

Patients and adrenocortical samples

DNA was extracted from peripheral lymphocytes from 18 members of the family, eight from generation II and ten from generation III, by standard methods. BMAH tissue specimens were obtained at surgery from patients II-1 and III-4 and were immediately frozen in liquid nitrogen and stored at -80°C . Tumor DNA was extracted from frozen tissues as described previously (27).

Analysis for mutations of ARMC5 gene

All six exons of the coding regions were screened for the presence of ARMC5 gene mutations. We used the primers previously described (18) and we designed two new sets of primers for the amplification of exon 1. The number of cycles and the annealing temperatures used for PCR amplification are detailed in [Supplemental Table 1](#) (see section on [supplementary data](#) given at the end of this article). The PCR products were separated on a 2% agarose gel and detected using a PhosphorImager. The amplicons were directly sequenced using Sanger sequencing in both directions (McGill University and Gnome Qubec, Montral, QC, Canada).

In silico analyses and comparison with controls in CARTaGENE database

We used the *in silico* software Mutation Taster (28) and SNAP (29) to predict the damaging potential of the genetic alterations found in lymphocyte and tumoral DNA samples. We compared the identified genetic alterations to data from the CARTaGENE project consisting of a cohort of 96 French-Canadian subjects (30).

Results

Following the diagnosis of two siblings with β -adrenergic/ V_1 -vasopressin responsive BMAH and clinical and sub-clinical CS, we hypothesized that other family members may be affected with BMAH (Fig. 2). Table 1 summarizes the clinical and biochemical characteristics of the nine family members found to be affected with BMAH.

Family history of the probands

The mother of the probands (II-1 and II-7) died at 82 years of age and suffered from Parkinson's disease. Their father died at 77 with high blood pressure, diabetes, central obesity, atherosclerotic cardiovascular disease, and chronic kidney failure. In addition to six living sisters, one sister died at 3 months of age from meningitis; another sister who suffered of hypertension and dyslipidemia died at 61 of cerebral metastasis from an unknown primary cancer. A paternal cousin was diagnosed at age 62 with cortisol-secreting adrenocortical carcinoma. No other tumors were known in the family.

Prospective clinical investigation of family members of generation II (II-2, II-3, II-5, II-6, II-8, and II-9)

Endocrine investigation in the six siblings identified three individuals aged 65, 59, and 56 years old (II-3, II-8, and II-9) with unsuspected sub-clinical CS secondary to BMAH in whom cortisol was stimulated by catecholamines and vasopressin both not by mixed meal, GnRH, TRH, and tegaserod/metoclopramide. The three other siblings of 67, 63, and 61 years old (II-2, II-5, and II-6) showed normal suppression following DST and no significant increase of cortisol during the upright posture and vasopressin tests. Aldosterone/renin ratios were normal in all cases.

Affected individuals in generation II (II-3, II-8, and II-9)

Patient II-3 ► This 65-year-old woman presented high blood pressure treated during the last 2 years with hydrochlorothiazide 25 mg and amlodipine 5 mg once daily and osteoporosis. Physical examination revealed facial plethora, sub-clavicular fat pads and a buffalo hump. Initial endocrine investigation showed abnormal 1-mg oral and 4-mg i.v. DST, but normal UFC (Table 1). Bilateral adrenal nodules were found on abdominal CT scan (Fig. 3). Cortisol secretion was stimulated by upright posture (289%) and vasopressin (355%). In light of past medical history of cardiac arrhythmias, insulin tolerance

test and isoproterenol infusion were not performed. Propranolol 40 mg four times during 3 days partially decreased the cortisol response to the posture test from 289 to 165%. No significant response of cortisol to tegaserod, LHRH or TRH tests were found. The patient is treated with nadolol 80 mg once daily and her UFC levels remained within the normal range.

Patient II-8 ► This 59-year-old woman had benign breast tumors, cholelithiasis, dyslipidemia, and pulmonary bronchiectasis. She was diagnosed at age 55 with osteoporosis and is a heterozygous carrier of factor V Leiden mutation. Physical exam was normal. She had sub-normal suppression to 1 mg-DST and aberrant cortisol response to upright posture test (278%) and to vasopressin stimulation (173%) but not to desmopressin (122%). Aberrant regulation of cortisol was confirmed with the insulin tolerance test (476%) and the isoproterenol infusion (377%). Under β -blockers, cortisol response to upright posture decreased only slightly from 278 to 219%. Abdominal CT-scan showed the presence of two 1-cm lesion on each adrenal gland. She has been treated with nadolol 40 mg once daily and is stable with sub-clinical CS.

Patient II-9 ► Their 56-year-old sister reported hypertension of 5 years duration treated with Irbesartan 300 mg/hydrochlorothiazide 25 mg. She was also found to have osteoporosis and underwent resection of a meningioma at age 54. On physical examination, there was diffuse obesity with a BMI of 28. There was abnormal suppression of cortisol to 1 mg-IV DST (580 nmol/l) and 4 mg-IV DST (463 nmol/l), while 24-h UFC was normal. CT showed bilateral adrenal enlargement (Table 1). CRH test (1 μ g/kg i.v.) increased ACTH from <2.2 to 9.5 pmol/l and cortisol from 458 to 1187 nmol/l, demonstrating an incomplete ACTH suppression. Abnormal secretion of cortisol was confirmed to the upright posture test (160%) and catecholamine-dependent increase of cortisol of 215% during the insulin tolerance test, of 159% during the treadmill stress test, and of 281% during the isoproterenol infusion. There was no cortisol response following desmopressin injection. The patient was treated with propranolol 40 mg twice daily only because she was not able to tolerate higher doses of β -blockers due to hypotension.

Non-affected individuals in generation II (II-2, II-5, and II-6) ► Individual II-2 (67-year-old woman) with type 2 diabetes and hypertension, had normal suppression to DST (1 mg overnight: 39 nmol/l and 4 mg IV: 45 nmol/l). Family member II-5 is a 63-year-old woman diagnosed

with hypertension, impaired glucose tolerance, obesity, hypothyroidism and symptomatic cholelithiasis. She had normal DST and UFC levels and a non-significant response in cortisol to the upright posture (128%). Individual II-6 was a 61-year-old woman with high blood pressure, benign breast nodules and osteoporosis who had had hysterectomy at age 34. She was under carbamazepine for trigeminal neuralgia and her physical exam showed no sign of CS, and UFC was normal. DST was slightly subnormal (62 nmol/l) under carbamazepine; following its discontinuation for 1 week, a 4 mg-IV DST was normal at 78 nmol/l. She showed no response to posture and vasopressin stimulations.

Prospective clinical investigation of family members of generation III

Among the 30 family members of generation III, 22 individuals (13 females and nine males) aged 25–46 consented for at least the 1 mg-DST. Thirteen individuals (nine females and four males) showed normal suppression. Among the nine individuals showing abnormal cortisol suppression to the 1 mg-DST, four subjects underwent further tests that confirmed sub-clinical CS in three subjects (two males of 46 (III-2) and 44 (III-10) years old, and one female 42-year-old (III-5)), and CS in one 44-year-old female (III-4). Among the affected individuals from generation III, there are one son (III-2) and two daughters (III-4 and III-5) of proband II-1 with sub-clinical (n : 2) and clinical (n : 1) CS and a son (III-10) of patient II-3 with a diagnosis of sub-clinical CS (Fig. 1). All these individuals had abnormal cortisol suppression to 4 mg-IV DST and were examined *in vivo* for the presence of aberrant cortisol response to catecholamines and vasopressin. Their aldosterone/renin ratios were within the normal limits.

Individual III-4 ► This 44-year-old gained 12 kg during the last 3 years. At screening, there was no suppression of cortisol to the 1 mg-DST (650 nmol/l) nor later to 4 mg-IV DST (615 nmol/l). UFC was increased at 772 nmol/day with undetectable plasma ACTH levels (<0.5 pmol/l). Similar to her father (proband II-1), her cortisol levels increased following posture test (144%), insulin tolerance test (148%), and isoproterenol infusion (216%) and after vasopressin (195%). Her adrenal CT-scan revealed multinodular adrenal glands (Table 1 and Fig. 3). She was treated with nadolol 40 mg once daily and her UFC decreased to 284 nmol/day. The response of cortisol to upright posture test remained significant (149%) although nadolol was increased up to 60 mg daily. Nadolol was replaced by

pindolol 5 mg twice daily because of bradycardia; One year later UFC increased to 1099 nmol/day with signs of CS. Upright posture tests were repeated under pindolol and clonidine 0.3 mg (cortisol increased from 658 to 1002 nmol/l, 152%) and the selective α_1 receptor antagonist prazosin (cortisol increased from 590 to 1302 nmol/l (221%)). A bilateral adrenalectomy was performed.

Individual III-2 ► This 46-year-old man had subnormal suppression to 1 mg DST (85 nmol/l). He had no complaints and his physical examination was normal. Sub-clinical CS mediated by catecholamines/vasopressin was confirmed by upright posture test (189%), the insulin tolerance test (595%), the isoproterenol infusion (327%), and vasopressin stimulation (269%). Unsuspected adrenal masses were seen at CT-scan (left nodule 15×26 mm; right nodule of 8 mm) (Fig. 3). He is currently treated with nadolol 20 mg once daily.

Individual III-5 ► This 42-year-old woman experienced 5 kg weight loss during the last year and has no high blood pressure or diabetes. Her physical exam was normal except for diffuse obesity with no CS stigmata. The investigation revealed the presence of sub-clinical CS with abnormal cortisol secretion to catecholamines-mediated stimuli (Table 1). Her CT-scan demonstrated bilateral adrenal Nodules. She was treated with nadolol 20 mg once daily as well.

Individual III-10 ► This 44-year-old man had no significant past medical history. His endocrine investigation revealed an unsuspected sub-clinical CS as well with catecholamine and vasopressin responses (Table 1). His CT-scan revealed bilateral adrenal lesions. A bone density revealed unknown osteoporosis with a T -score of -2.6 in L1–L4 areas. He is also treated with nadolol.

Mutational analysis of ARMC5 gene

A heterozygous germline *ARMC5* non-sense mutation c.327_328insC (p.Ala110Argfs*9) was identified in lymphocyte DNA of the index case II-1 (Fig. 2). The *in silico* modeling of protein function of this *ARMC5* mutation was predicted as damaging using Mutation Taster (28) and non-neutral using SNAP (29) predicting that it is disease-causing. This genetic alteration was not found in 96 French-Canadian controls. Two additional somatic *ARMC5* variants were found in the adrenal tissue of patient II-1; i) the heterozygote silent mutation c.288C>G; p.A96A and ii) a somatic non-sense mutation

C.2029G>T (p.E677X). This last pathogenic somatic mutation supports the hypothesis of the tumor suppressor role of *ARMC5*. The daughter (III-4) of II-1 who was affected by cortisol-secreting BMAH and CS at age 44 carries the germline heterozygous c.327_328C (p.Ala110Argfs*9) *ARMC5* mutation as well, but in addition carries a germline heterozygous p.A705V *ARMC5* genetic alteration reported as a gene polymorphism (rs11150624) (Table 2). This variant was found in 50% of our cohort of 96 French-Canadian individuals (30).

The germline pathogenic *ARMC5* non-sense mutation c.327_328insC (p.Ala110Argfs*9) was found in nine individuals with clinical or subclinical CS, in 2/5 individuals with abnormal suppression to dexamethasone at initial investigation and 1/6 individuals (this individual was 40 years old) (from generation III) with normal clinical screening tests.

Discussion

To our knowledge, we describe the largest family with hereditary BMAH characterized extensively for the presence of aberrant hormone receptors, including nine affected members with clinical or sub-clinical CS. All the affected members expressed the same β -adrenergic/V₁-vasopressin-responsive cortisol secretion phenotype that was extensively characterized previously *in vivo* and *in vitro* in the propositus (24). The first BMAH family with β -adrenergic/vasopressin-responsive cortisol secretion was described in 2002 by Miyamura *et al.* (5) and included an affected mother and son, but no other members of the family were investigated. Here, we identified sub-clinical CS in three females and one male and clinical CS in a male from generation II. Moreover, unexpected sub-clinical CS was diagnosed prospectively in two males and one female and clinical CS in one female from generation III. Among all the patients investigated in this study, 5/8 (63%) living individuals from generation II and 9/22 (41%) individuals tested from generation III showed at least abnormal suppression to the 1 mg DST. Although diagnosis of BMAH was confirmed in all 5/5 subjects in generation II, only 4/9 individuals from generation III with an abnormal 1 mg DST have undergone complete confirmatory tests for the presence of BMAH. As described previously in other families, we observed an autosomal dominant pattern of transmission of the disease (21).

Our prospective familial clinical screening of BMAH following diagnosis of the index cases was conducted several years before the identification of the gene implicated in familial BMAH. The diagnosis of hereditary

Table 2 Summary of *ARMC5* genetic alterations found in lymphocyte and tumoral DNAs of patients II-1 and III-4.

Patients	Transcript ID	DNA change	Protein change	G or S	Chromosome position	SNP	Mutation Taster	Controls n=96 FC
II-1	ENST00000268314	c.327_328insC heterozygote	p.A110Rfs*9	G	31471172–31471173	No	Disease-causing	0%
	ENST00000268314	c.288C>G heterozygote	p.A96 A	S	31471133	No	Polymorphism	0%
	ENST00000268314	c.2029G>T heterozygote	p.E677X	S	31477431	No	Disease-causing	0%
III-4	ENST00000268314	c.327_328insC heterozygote	p.A110Rfs*9	G	31471172–31471173	No	Disease-causing	0%
	ENST00000457010	c.2114C>T heterozygote	p.A705V	G	31476458	rs11150624	Polymorphism	50%

G, germline; S, somatic; FC, French-Canadian.

Clinical Study	I Bourdeau and others	ARMC5 mutation in familial BMAH	174:1	94
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BMAH will now be facilitated as an *ARMC5* mutation has been found in an index case. Patients with apparently sporadic BMAH should be offered genetic counseling for germline *ARMC5* gene analysis and because BMAH has an autosomal mode of transmission, if the proband is found to carry mutations in *ARMC5*, genetic testing should be offered to all first-degree relatives. However, some families with BMAH do not carry *ARMC5* mutations and are likely carriers of another genetic mutation not yet identified. Thus, clinical screening with 1 mg-DST and imaging as utilized in this study will be necessary for BMAH families with no identified germline mutation causing disease. Our prospective clinical screening strategy allowed us to identify six subjects with unsuspected sub-clinical CS and one female with unknown CS and BMAH. As observed by Alencar *et al.* (21) and Elbelt *et al.* (22), the 1 mg-overnight DST is the most sensitive screening method and far superior to UFC for the identification of subjects with unsuspected secreting BMAH. Considering the long-term deleterious effects of hypercortisolism, the early diagnosis of cortisol-secreting BMAH is of importance. Although the surgical treatment of sub-clinical CS remains controversial, the identification of aberrant-regulation of cortisol by various receptors including β -adrenergic and V_1 -AVPR allowed us to treat medically several patients with sub-clinical or clinical CS with β -blockers. For example, in proband II-1, adjuvant therapy with β -blockers after a unilateral adrenalectomy enabled the long-term control of hypercortisolism, and avoided bilateral adrenalectomy with the risk of acute adrenal insufficiency (24). Although the long-term effects of β -blockers in subjects with aberrant combined β -AR and V_1 -AVPR with sub-clinical CS remain to be evaluated, in the 18 years' follow-up of proband II-1 stabilization of adrenal gland size and secretion with long-term medical treatment were confirmed. However, the coexistence of more than one aberrant GPCR, including AVPR1 in this family, may limit the success of monotherapy with β -blockers, as shown by the incomplete abolition of cortisol response to upright posture and the requirement of bilateral adrenalectomy in patient III-4. Based on *in vitro* data demonstrating the frequent expression of alpha 2A adrenergic receptor (ADRA2A), we examined whether cortisol was regulated by clonidine or prazosin, but we found no such response in individual III-4 (31).

Other BMAH kindreds were described with adrenal aberrant receptors. Imöhl *et al.* described a 44-year-old male and his mother affected by catecholamine-dependent BMAH (7). Lee *et al.* (8) described two sisters (46 and 58 years old) with BMAH, meningiomas, and ectopic

expression of vasopressin V_1b and V_2 receptors. A French family with BMAH was found to have aberrant 5-HT₄ and vasopressin adrenal receptors in three siblings (two females of 54 and 56 years old, and one 57-year-old male) and their 81-year-old father (9). Very recently, in an Australian kindred carrying an *ARMC5* mutation, vasopressin-responsive BMAH was present in seven members of the family (23); two other Australian BMAH families with partial endocrine investigation suggested vasopressin responsiveness in a father and a son in one family, and in three siblings in the second family (10). In contrast to the family in this study, these authors found no correlations between DST or vasopressin and the diagnosis of BMAH ascertained by adrenal imaging. Our study of a large Brazilian family with BMAH demonstrated that the aberrant responses to vasopressin or metoclopramide were not consistently present in all affected individuals (15).

As found recently in other BMAH kindreds (21, 22, 23), we identified a damaging germline non-sense c.327_328insC (p.Ala110Argfs*9) mutation of *ARMC5* in all the clinically affected members of our kindred. As expected, some mutation carriers are still too young to express clinical disease and a longer follow-up will be necessary to assess the penetrance of the genetic alteration. Very recently, Elbelt *et al.* (22) described a German family with a heterozygous c.323_324insC *ARMC5* mutation which is the same mutation that we report here, although according to the recommendations by the Human Genome Variation Society, it should be preferentially named c.327_328insC. In contrast to studies on sporadic cases (18, 19), we had access to only two BMAH tissues and found a second additional somatic *ARMC5* mutation in the BMAH tissue as reported by Gagiardi *et al.* (23). In addition to the causative germline mutation, we found a second germline heterozygous A705V *ARMC5* alteration in the daughter who presented a very early onset of severe BMAH (even though a second somatic mutation was not found); although this latest genetic alteration reported as a gene polymorphism (rs11150624) does not affect the main *ARMC5* transcript but instead the *ARMC5* isoform b precursor, it is intriguing that an isolated non-damaging alteration of a potential *ARMC5* transcript accelerates the clinical evolution of the disease. It can also be speculated that other somatic events can accelerate the phenotype such as those identified recently in the highly conserved methyltransferase domain of DOT1L, in the histone deacetylase gene *HDAC9*, which were not examined in this patient's tissues (32). The paternal cousin of the proband developed an adrenocortical carcinoma but was not a carrier of the *ARMC5* mutation identified.

We conclude that this ACC was coincidental or at least not due to this *ARMC5* gene alteration.

As *ARMC5* is a widely expressed gene in a large number of human tissues and functions as a tumor-suppressor gene, it is relevant to investigate whether other tumors develop in patients or families carrying inactivating *ARMC5* mutations (33). Intracranial meningiomas were present in a Korean family with BMAH (8) and in 43% of the Brazilian family members with germline *ARMC5* mutations and BMAH (21), while they are diagnosed in only 0.9% of the general population after the fourth decade of life. Very recently, in the German family carrying the *ARMC5* c.323_324insC (p.Ala110Argfs*9) mutation a damaging somatic *ARMC5* mutation was shown in a concomitant meningioma but not in a pancreatic tumor found in an affected subject (22). In this family, there was only one case of resected meningioma patient II-9 (no tissue available for analysis) and no history of other neoplasias.

This is a first study in which a familial BMAH family is extensively characterized for the presence of aberrant hormone receptors associated with an *ARMC5* mutation. The adrenocortical expression of high affinity binding sites compatible with β 1- or β 2-adrenergic receptors efficiently coupled to steroidogenesis in the BMAH tissues in these patients, but not in normal adrenal cortex indicated the ectopic nature of this receptor (5, 24). The apparent genetic transmission of the same receptor phenotype in all affected family members supported the hypothesis of a primary role of aberrant receptors in the pathophysiology of BMAH; however, the causal role of *ARMC5* mutations in this and other BMAH families, suggests that the aberrant GPCRs are secondary events, although the mechanisms leading to their altered expression remain to be elucidated. Further studies will be necessary to clarify whether there is any relation between *ARMC5* mutations and aberrant GPCR or autocrine production of ACTH in BMAH tissues.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-15-0642>.

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.

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Author contribution statement

Conception and design: P Hamet, J Tremblay, S Oble, A Lacroix, and I Bourdeau. Collection and assembly of data: I Lévesque, F Magne, S Oble, K Caceres, S Nolet, M C B V Fragoso, A Lacroix, and I Bourdeau. Data analysis and interpretation: S Oble, F Magne, K Caceres, P Hamet, J Tremblay, P Awadalla, A Lacroix, and I Bourdeau. Manuscript writing: A Lacroix and I Bourdeau. Final approval of manuscript: all authors.

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