Primary macronodular adrenal hyperplasia (PMAH) can be generated by a new ARMC5 germline variant (c.52C>T (p.Gln18X))

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Abstract. Primary macronodular adrenal hyperplasia (PMAH) is a rare cause of Cushing syndrome (CS). In many cases of the PMAH family, variant in ARMC5, a putative tumor suppressor gene, are thought to induce the disease. The purpose of this study was to report a large Chinese family, in which a new germline heterozygous variant of ARMC5 (c.52C>T (p.Gln18X)) was found. A 64-year-old female patient (proband) was admitted to the hospital due to bilateral adrenal masses. In order to clarify the nature and function of adrenal masses, the proband completed several relevant screening tests of the adrenal function. After an ectopic receptor screening test and genetic testing, a new ARMC5 gene variant was found that might had led to the occurrence of PMAH. Because of its characteristic of autosomal dominant inheritance, the proband’s relatives were recommended to conduct the genetic test. We collected the family members’ genetic information, in which have 27 individuals, the proband tested the whole exon sequence, and 12 participants tested the Sanger sequence. Finally, 7 individuals were found have the same germline variant of ARMC5 as the proband. Subsequent computer analysis predicted that the variant significantly impaired protein function and resulted in inactivation of ARMC5. We found a new germline ARMC5 variant (c.52C>T (p.Gln18X)), which may induced PMAH. ARMC5 sequencing can improve the identification of clinical forms of PMAH and allow early diagnosis of the disease.

Key words: Primary macronodular adrenal hyperplasia, Cushing syndrome, ARMC5, Gene variant, Sequencing

PRIMARY MACRONODULAR ADRENAL HYPERPLASIA (PMAH) is a subtype of Cushing syndrome (CS) or subclinical Cushing syndrome (SCS) characterized by multiple nodular hyperplasia of primary bilateral adrenal cortices [1-3]. The prevalence of bilateral PMAH in CS is reported to be <1% [4]. The symptoms of PMAH develop slowly, and the disease has a long incubation period so that the timing of diagnosis is relatively late. Therefore, there is a higher prevalence of PMAH in elderly people with SCS. Generally, the rate of diagnosis is the highest at the age of 40–70 [5, 6].

Although the pathophysiology of PMAH has not been fully elucidated, previous studies have shown that abnormal G-protein-coupled membrane receptors are expressed in proliferative adrenal cells that regulate cortisol synthesis [7]. Mutations in various genes and somatic cells have also been reported to be involved in PMAH. Genes associated with PMAH include PDE11A (phosphodiesterase 11A) [8], GNAS (stimulating G protein alpha subunit) [9, 10], APC (colony adenomatous polyps) [11, 12], FH, fumaric acid hydratase; ACTH, adrenocorticotropic hormone; UFC, urine-free cortisol; SFC, serum-free cortisol; CT, computed tomography; ACTH-COR, adrenocorticotropic hormone and cortisone rhythm; MRI, magnetic resonance imaging; WES, whole exome sequencing; EDTA, ethylenediaminetetraacetic acid; LDDST, low-dose dexamethasone suppression test; SNP, single nucleotide polymorphisms; ACA, adrenocortical adenoma; ACC, adrenal cortical cancer; BAH, bilateral adrenal hyperplasia; AUMAH, ACTH-independent macronodular adrenal hyperplasia; PPNAD, primary pigmented nodular adrenocortical disease; IMAD, isolated macronodular adrenocortical disease; GIP, gastric inhibitory peptide; LH, luteinizing hormone; AVP, arginine vasopressin; 5-HT, serotonin; CA, catecholamine; MC2R, melanocortin 2 receptor
polyps) [10, 11], FH (fumaric acid hydratase) [10, 12] and others [13].

In 2013, Assié, et al. [14] used whole-genome sequencing and single nucleotide polymorphism array analysis to find a variant in the ARMC5 gene on the 16p chromosome for the first time in a PMAH family, among which 55% (18/33) of family members underwent surgery. In the 18 patients with ARMC5 variant, two kinds of ARMC5 alleles were mutated: one was a germline variant and the other was a somatic cell. Four of these cases had germline mutations with nodule-specific secondary ARMC5 variant. This indicates that the role of ARMC5 is consistent with Knudson’s two-hit hypothesis: it is a tumor suppressor gene with a tendency to induce the development of tumors. In the case of a previously existing germline inactivation mutation in one allele, the other allele undergoes a secondary somatic inactivation mutation. Subsequently, several studies have reported ARMC5 variants in 21%–44% of patients with bilateral PMAH [15-19]. A series of clinical genomic sequencing studies have shown that ARMC5 variants play an important role in the development of bilateral PMAH adrenal tumors [20, 21], which usually manifest as adrenal hyperplasia with multiple nodules [15-17, 22]. Therefore, the bilateral nature of the disease and ARMC5 variants suggest the heritability of bilateral PMAH.

In this study, complete exon sequencing of a PMAH proband and her family was performed. The suspected ARMC5 variant site of PMAH was screened in combination with bioinformatics, and the variants in the family members were verified by Sanger sequencing. A new ARMC5 variant was found, which will provide a basis for the genetic pathogenesis of PMAH.

**Materials and Methods**

**Patient**

A 64-year-old female patient (proband) was admitted to the Department of Endocrinology, the First Affiliated Hospital of China Medical University in 2018, with “20 years of hypertension, 3 years of adrenal masses and poor blood pressure control for 2 months”. However, the proband had no Cushing’s signs such as moon face, central obesity, and buffalo hump. After examination, the main diagnoses were: adrenocorticotropic hormone (ACTH) independent SCS, bilateral adrenal masses, and hypertension (secondary). On genetic testing, the patient was found to have PMAH. The patient’s family consists of four generations, including 27 members, of which the first generation, the proband’s parents [I-2 and I-3] and the proband’s stepfather [I-1] had died; three others could not be included in the study because of objective conditions, the nephew of the proband [III-1, 50-year-old], the grandniece of the proband [IV-1, 20-year-old], and the nephew of the proband [IV-6, 2-year-old]. Twenty people were tested, including eight members who have gene variants. In the second generation are the proband [II-4, 64-year-old], the brother of the proband [II-2, 70-year-old], and the sister of the proband [II-7, 62-year-old]; in the third generation are the nephew of the proband [III-3, 46-year-old], the daughter of the proband [III-5, 38-year-old], and the niece of the proband [III-7, 33-year-old]; the fourth generation includes the grandnephew of the proband [IV-2, 22-year-old] and the granddaughter of the proband [IV-3, 8-year-old]. No close relatives of the family members were married, and the genetic map of the family members is shown in Fig. 1. All family members included in the study provided signed informed consent (underage family members were signed for by their parents).

**Diagnostic criteria**

William, et al. [23] recommend that patients with the following conditions can be diagnosed as having bilateral PMAH: (1) Relevant clinical manifestations and signs of Cushing syndrome: concentric obesity, diabetes or impaired glucose tolerance, hypertension, osteoporosis, etc., clinical manifestations caused by negative nitrogen balance (muscle atrophy throughout the body, which is more pronounced in the extremities; the skin is thin and transparent in the lower abdomen, outer hips, inner thighs, around armpits and breasts, etc., due to reduced collagen; typical symmetrical purple lines on the skin; skin capillaries have increased fragility and are prone to ecchymosis, which is more common in the upper arms, back of hands, and inner thighs; skin wounds do not heal easily). (2) Laboratory tests: plasma cortisol levels and circadian rhythm disorders; 24h urine-free cortisol (UFC) and serum-free cortisol (SFC) increased; low-dose dexamethasone suppression tests show partial or no inhibition; ACTH inhibition and ACTH excitability test negative. (3) Imaging examination: adrenal computed tomography (CT) shows obvious hyperplasia of bilateral adrenal glands, with multiple nodules of different sizes (diameter ≥10 mm), “ginger-like” characteristic changes. (4) Pathology shows adrenal nodular hyperplasia.

**Laboratory inspection**

Relevant laboratory tests for probands include: adrenocorticotropic hormone and cortisone rhythm (ACTH-COR), low-dose dexamethasone suppression test, high-dose dexamethasone suppression test, and ectopic receptor screening tests (posture test, mixed meal test, Gonaray Forest test, metoclopramide test, posterior pituitary hormone test).
Imaging inspection
For the differential diagnosis of multiple endocrine neoplasia-1, the adrenal enhancement CT and pituitary magnetic resonance imaging (MRI) inspection was performed on the probands.

Whole exome sequencing (WES)
A sample of 5 mL peripheral blood per individual was collected from the proband and 12 relatives mentioned, anticoagulated with ethylenediaminetetraacetic acid (EDTA), and stored at −80°C. Later, whole genome DNA was extracted. The proband’s DNA samples were sequenced at different depths. A 400 bp insert size sequencing library was constructed from 2 μg genomic DNA using the truseq DNA sample preparation kit (Illumina), and then sequenced on an Illumina hiseq sequencing platform and the whole exon capture platform of the Roche probe group. The sequencing depth and coverage of each sample met the requirements of bioinformatics data analysis. Illumina sequencing data were located to the human reference genome using BWA (0.7.12-r1039) software and compared with the standard genome. For rare variants of the probands, we used ANNOVAR (SDate: June 17, 2015) software to annotate mutated sites with dbSNP, Clinvar, ExAC, thousand genomes and other databases. The adrenal-associated genetic disease genes (NR0B1, MC2R, MRAP, SF1, ARMC5, etc.) related to the clinical symptoms of the patients were analyzed, and a mutation with unknown clinical cause was detected. Sanger sequencing was performed on the peripheral blood samples of the 12 family members using an ABI 3730 sequencer to verify the co-segregation of genotype and phenotype. Sanger sequencing verification of the mutation site involved PCR, Primer Premier 6.0 was used to design primers for verification:

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\text{ARMC5-1F; 5'-CGAGAACTACAACTTCCGACTT-3'},
\text{ARMC5-1R; 5'-CACGTTATTCCGGGATAGG-3'}.\]

BigDye® Terminator v3.1 and ABI 3730 sequencing verification using the peripheral blood of the proband and her relatives. The DNA sequence of ARMC5 was queried in the Ensembl Genome Browser, and Primer premier 6.0 was used to design primers for verification: 

\[
\text{ARMC5-1F; 5'-CGAGAACTACAATTCCGCAGTT-3'},
\text{ARMC5-1R; 5'-CACGTTATTCCGGGATAGG-3'}.\]

Results
Laboratory inspection
Laboratory test results of the proband showed the loss of cortisol circadian rhythm, and ACTH was suppressed. The low-dose dexamethasone suppression test
(LDDST) showed that cortisol was not be inhibited (COR = 258.1 nmol/L, normal range <50 nmol/L). The above results suggest a diagnosis of ACTH-independent SCS. In addition, the related ectopic receptor screening test was positive. In contrast to the positive body position test results (Table 1), the mixed meal test, gonarelin test, metoclopramide test, and vasopressin load test were all negative.

Imaging examinations
Adrenal CT showed bilateral adrenal enlargement, irregular morphology, and nodules (Fig. 2). The maximum lateral diameter of the right adrenal gland was 3.0 × 2.8 cm. The maximum lateral diameter of the left adrenal gland was 3.5 × 2.7 cm. The CT value is 5 HU. The enhanced scan showed irregular enhancement of both adrenal glands, and nodular enhancement. The surrounding fat gap was clear. Multiple small dot-shaped high-density shadows could be seen in both kidneys. A circular cystic low-density shadow could be seen in the right kidney, with a size of about 1.7 × 1.3 cm. Multiple small circular cystic low-density shadows were observed in the liver, and multiple spot-shaped high-density shadows in the gallbladder. The gallbladder wall was thickened. No significant lymph nodes were seen behind the peritoneum. Imaging conclusions: bilateral adrenal gland space-occupying lesions; multiple kidney stones; right kidney cysts; liver cysts; gallstones; cholecystitis. No significant abnormalities were found in the pituitary MRI.

Whole exome sequencing
The proband’s full exon sequencing and bioinformatics analysis gave the following results. Sanger sequencing results showed that there was a c.52C>T variant on the ARMC5 gene locus NM_001105247 Exon1. The occurrence of heterozygous variants (Fig. 3) resulted in amino acid changes (p.Gln18X), so that we thought it might lead to the clinical phenotype of the proband. In this case, we considered that this heterozygous variant at the ARMC5 mutation site caused the disease in the family. WES of the germline DNA discovered this novel heterozygous germline ARMC5 variant (c.52C>T, p.Gln18X) and in silico analysis predicted that the variant significantly impaired protein function, resulting in inactivated ARMC5 (Supplementary Table 1). Subsequently, WES identified other 16 somatic single nucleotide polymorphisms (SNPs)/insertion–deletion

Table 1  Laboratory inspection

|                      | ACTH (pg/mL) | COR (nmol/L) |
|----------------------|--------------|--------------|
| Rhythm 8:00          | 2.55         | 391.7        |
| Rhythm 15:00         | 1.02         | 258.5        |
| Rhythm 24:00         | 1            | 169.7        |
| LDDST 8:00           | 1            | 258.1        |
| Supine 1 h           | 3.19         | 364.9        |
| Stand 1 h            | 3.19         | 453.9        |
| Stand 1.5 h          | 5.20         | 550.8        |
| Stand 2 h            | 4.86         | 584.4        |

The patient showed ACTH-COR rhythm disorder, and ACTH was suppressed. The low-dose dexamethasone suppression test (LDDST) showed that cortisol was not inhibited (COR = 258.1 nmol/L, normal range <50 nmol/L). The related ectopic receptor screening test (body position test) was positive.

Fig. 2  Adrenal CT showed bilateral adrenal enlargement, irregular morphology, and nodules. The maximum lateral diameter of the right adrenal gland was 3.0 × 2.8 cm, the maximum lateral diameter of the left adrenal gland was 3.5 × 2.7 cm. The enhanced scan showed irregular enhancement of both adrenal glands, and nodular enhancement. The surrounding fat gap was clear. Multiple small dot-shaped high-density shadows could be seen in both kidneys. A circular cystic low-density shadow could be seen in the right kidney, with a size of about 1.7 × 1.3 cm.
(indel) mutations. None of these mutations were related to CS.

**Discussion**

In this study, the proband presented with hypertension and was diagnosed with non-ACTH-dependent subclinical CS during examinations related to secondary hypertension, and it was found that the proband had bilateral space-occupying lesions of the adrenal glands, observed to be nodular hyperplasia. The genetic testing of the patients and her family showed a heterozygous variant in c.52C>T at the **ARMC5** gene locus NM_001105247 Exon1 which resulted in amino acid changes (p.Gln18X). Later on, we collected the disease history of proband’s family members. We found that the proband’s sister (II-7, 62-year-old) had adrenal disease the same as the proband and the gene test result was positive as well. We considered that the proband’s sister’s adrenal disease was related to the germline ARMC5 mutations very likely. As for other family members of the proband, we found that the proband’s younger brother (II-6, 57-year-old) and nephew [III-3, 46-year-old] had high blood pressure. In addition, other family members are all healthy. All above evidences proved that the ARMC5 gene variant maybe the reason of PMAH in this Chinese family.

It is known that 20%–30% of CS is caused by an increase in primary adrenal cortex hormones, and adrenocortical adenoma (ACA) with increased cortisol production accounts for 10%–15%, of which adrenal cortical cancer (ACC) is less than 5%. Bilateral adrenal hyperplasia (BAH) accounts for approximately 10% of adrenal-derived CS. The most common forms of BAH are ACTH-independent macronodular adrenal hyperplasia (AIMAH, similar to bilateral PMAH), primary pigmented nodular adrenocortical disease (PPNAD), and isolated macronodular adrenocortical disease (iMAD). The diameter of adrenal nodules in AIMAH is usually greater than 1 cm, while the diameter of adrenal nodules in PPNAD and iMAD is less than 1 cm. In this study, the maximum lateral diameter of the right adrenal gland of the proband was 3.0 × 2.8 cm, and the maximum lateral diameter of the left adrenal gland was 3.5 × 2.7 cm. Therefore, PMAH was highly suspected.

At present, the etiology of AIMAH is not clear. Several studies have confirmed that AIMAH can be caused by other factors than ACTH, such as gastric inhibitory peptide (GIP), β2-adrenaline, luteinizing hormone (LH), arginine vasopressin (AVP), serotonin (5-HT) and catecholamine (CA); when abnormally expressed in the adrenals these receptors can cause AIMAH. Louis, *et al.* [24] found that ectopic adrenal receptors may promote cortisol secretion through the activation of the protein kinase A pathway and membrane channels via cAMP, and then promote the occurrence of AIMAH. Therefore, the proband underwent the ectopic receptor screening test. Louiset, *et al.* [25] also studied the molecular, immunohistochemical, and pharmacological characteristics of vasopressin receptors in the cells of three patients with AIMAH, and found that the expression of functional ectopic V(2) receptors and repression of eutopic V(1a) receptor can coexist in some hyperplastic cortico-steroidogenic tissues. A study of AIMAH found that when the patient was in an upright position or suffering from hypoglycemia (insulin-induced), blood catecholamine levels increased, and cor-

![Image](image-url)
tisol levels also increased. β-Adrenergic agonists or isopropyl adrenaline stimulate secretion of blood aldosterone and cortisol, and propranolol reduces blood cortisol [26, 27]. At present, there are a variety of ectopic receptor screening tests, such as the body position test, mixed meal test, Gonarelin test, metoclopramide test, and posterior pituitary hormone test. The position test [28, 29] is a simple and efficient detection method for screening for AIMAH. When the body position is changed, there is a significant change in the secretion of cortisol. When the patient is in the standing position and cortisol secretion increases >125%, ectopic receptor may be expressed. When the patient is in the standing position and cortisol secretion increases >150%, the expression of adrenal ectopic receptors is highly suspected. In this study, the corticosteroid level of the proband increased can autonomously secret more cortisol, and have larger abnormal secretion of cortisol. In 2015, Zilbermint, adrenocortical nodules than patients without ARMC5 were unable to detect their laboratory inspection and imaging examination except proband. This may because they still have not have obvious clinical symptoms, and the associated tests consume time and energy. But they are willing to come if the symptoms appear. Second, the proband refused surgical treatment, and we were unable to detect the expression of related genes in the tumor tissue. Third, a prolonged follow-up period is needed to evaluate the condition of macronodular adrenal hyperplasia.

**Conclusions**

Analysis of ARMC5 gene variants can improve the identification of clinical forms of PMAH, and can be used to diagnose the disease at an early stage. It is recommended that patients with suspected PMAH undergo early ectopic receptor screening tests and ARMC5 gene testing.

**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.

**Ethics Statement and Informed Consent**

Informed consent was obtained from all individual participants included in the study. All the individual participants consented to the publication of medical data (including figures from diagnostic imaging results and from histological examination results). This study was performed following the approval of the institutional review board at Medical Science Research Ethics Committee of the First Hospital of China Medical University and the 1964 Helsinki declaration and its later amendments or comparable ethical standards.
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