Unanswered Questions in the Genetic Basis of Primary Aldosteronism

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Key words
aldosterone-producing adenoma, bilateral adrenal hyperplasia, genetics, familial hyperaldosteronism

received 04.09.2017
accepted 14.09.2017

Bibliography
DOI https://doi.org/10.1055/s-0043-120066
Published online: 24.10.2017
Horm Metab Res 2017; 49: 963–968
© Georg Thieme Verlag KG Stuttgart · New York
ISSN 0018-5043

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ABSTRACT
Over the past six years, the genetic basis of a significant fraction of primary aldosteronism (PA) cases has been solved. Breakthrough discoveries include the role of somatic variants in the KCNJ5, CACNA1D, ATP1A1, and ATP2B3 genes as causes of aldosterone-producing adenomas (APAs), and the recognition of three novel hyperaldosteronism syndromes with germline variants in the KCNJ5, CACNA1D, and CACNA1H genes. The description of somatic variants in CACNA1D and ATP1A1 in aldosterone-producing cell clusters (APCCs) suggests that these clusters are precursors of some aldosterone-producing adenomas. Yet, a number of questions remain unanswered. These include the genetic basis of about 40% of APAs without somatic variants in known genes. Do technical issues explain this finding, or are the unexplained APAs due to somatic copy number variation or rare variants in thus-far undiscovered genes? Similarly, the role of CTNNB1 (beta catenin) variants in APA pathogenesis is still unclear. The major question to be solved is the genetic basis of bilateral adrenal hyperplasia (BAH). Is BAH due to the bilateral occurrence of APCCs, to germline variants, or perhaps due to unknown serum factors? Lastly, the etiology of unsolved cases of apparently familial hyperaldosteronism remains to be discovered. It is expected that genetic studies over the next few years will lead to answers to at least some of the questions raised.

Abbreviations
ACTH Adrenocorticotropic hormone
APA Aldosterone-producing adenoma
APCC Aldosterone-producing cell clusters
ATP1A1 ATPase Na+/K+ Transporting Subunit Alpha 1
ATP2B3 ATPase Plasma Membrane Ca2+ Transporting 3
BAH Bilateral adrenal hyperplasia
CACNA1D Calcium channel, voltage-dependent, L type, alpha 1D subunit
CACNA1H Calcium channel, voltage-dependent, T type, alpha 1H subunit
CNV Copy number variation
CYP1B1 Cytochrome P450, family 11, subfamily B, polypeptide 1
CYP1B2 Cytochrome P450, family 11, subfamily B, polypeptide 2

FH Familial hyperaldosteronism
GRA Glucocorticoid-remediable aldosteronism
KCNJ5 Potassium channel, inwardly rectifying subfamily J, member 5
PA Primary aldosteronism
PASNA Primary aldosteronism, seizures, and neurologic abnormalities
SNV Single nucleotide polymorphism

Introduction
Primary aldosteronism (PA) is considered the most common cause of secondary hypertension. A recent study reported a prevalence of about 6% [1] in primary care, with higher prevalence among severely hypertensive cases. The prevalence in hypertension referral...
centers is about 6–10 % [2–4]. PA is characterized by excessive production of the adrenal steroid hormone aldosterone despite suppressed levels of its upstream regulator, the aspartyl protease renin, and normal or low serum potassium levels. Aldosterone-producing adenomas (APAs, with single or multiple nodules [5]) account for about 30 % of cases, and bilateral adrenal hyperplasia for about 60 % [4]. Diffuse unilateral hyperplasia is less common [6], whereas malignancy [7] and familial hyperaldosteronism (FH) [8] are considered rare.

Major progress has been made in understanding the genetic basis of PA over the past six years, but many questions remain unanswered [9]. This brings to mind U.S. Secretary of Defense Donald Rumsfeld’s famous statement: “…as we know, there are known knowns; there are things we know we know. We also know there are known unknowns; that is to say we know there are some things we do not know. But there are also unknown unknowns – the ones we don’t know we don’t know” [10]. These three aspects of the genetics of PA will be covered in this review.

The Known Knowns

About 40 % of APAs are caused by one of two somatic (tumor specific) variants in the KCNJ5 gene, and other variants are very rare [11–13]. KCNJ5 encodes an inward rectifier potassium channel. The disease-causing variants are located within or close to the selectivity filter of the channel and cause abnormal sodium permeability. Sodium influx through mutant channels leads to cellular depolarization, activation of voltage-dependent calcium channels, calcium influx and activation of the pathways that regulate aldosterone production [11, 14]. Approximately 10 % of tumors carry somatic variants in the CACNA1D gene, encoding a voltage-gated calcium channel [13, 15, 16]. These variants directly cause increased calcium influx through the mutant channel. Mutations in the Na⁺/K⁺ ATPase subunit gene ATP1A1 account for approximately 5 % of tumors, and mutations in the plasma membrane Ca²⁺-ATPase gene ATP2B3 for a small percentage (about 2 %) [13, 16, 17]. These variants cause abnormal permeability to sodium or proton ions, leading to depolarization similar to KCNJ5 mutations [16, 18]. Lastly, mutations in the CTNNB1 gene encoding beta catenin are found in 2–5 % of tumors [19–21].

Important insight into the origin of APAs came from the description of subcapsular aldosterone-producing cell clusters (APCCs) that are found in normal adult adrenal glands. These clusters also carry somatic variants in the CACNA1D and ATP1A1 genes [22] (► Fig. 1), suggesting that they represent precursors of APAs carrying such variants [23].

Familial aggregation of primary aldosteronism was initially reported in a father and his son with glucocorticoid-remediable aldosteronism (GRA, FH-I) [24]. GRA is due to unequal cross over between CYP11B1, encoding 11β-hydroxylase involved in cortisol production under the control of adrenocorticotropic hormone (ACTH), and CYP11B2, encoding aldosterone synthase [25]. This leads to ACTH-dependent aldosterone production in the zona fasciculata. Following the discovery of somatic KCNJ5 and CACNA1D mutations in APAs, inherited or de novo germline variants in the same genes were identified in FH-III [11, 26, 27] and PA with seizures and neurologic abnormalities (PASNA) syndrome [15], respectively. Lastly, germline CACNA1H variants, which cause gain of function of the encoded calcium channel, were discovered in FH-IV [28].

The Known Unknowns

Despite the significant progress in understanding PA, several questions remain unanswered. One of the most obvious “known” questions is the genetic basis of the about 40 % of aldosterone-producing adenomas without mutations in KCNJ5, CACNA1D, ATP1A1, ATP2B3, and CTNNB1. Several not mutually exclusive explanations come to mind.

The first explanation is of technical nature. APAs can demonstrate intra-tumor heterogeneity. Some tumor areas can be positive for aldosterone synthase expression by immunohistochemistry, whereas others are not, and variants are only found in aldosterone synthase positive areas [29]. Because not all tumor areas may carry disease-causing variants and biopsies can be contaminated with surrounding normal tissue and/or stroma, variant allele frequencies can be below Sanger sequencing detection levels. This explains why variants are occasionally found by next-generation sequencing in tumors that are negative by Sanger sequencing [19]. Analysis of large genes with variants scattered throughout the gene, such as CACNA1D, is particularly challenging by Sanger sequencing, and many studies have only analyzed part of the gene, so mutation frequencies may have been underestimated. APAs are also not always single circumscribed tumors. Surrounding hyperplasia and multinodular glands have also been described [5, 19], and in the absence of aldosterone synthase immunohistochemistry, it can be difficult to distinguish non-producing nodules from those producing aldosterone. Taken together, studies performed on tissue biopsies without immunohistochemistry against aldosterone synthase may have underestimated “true” mutation frequencies.

The second explanation would be that variants in additional genes that are only mutated in a small fraction of samples have so far evaded detection by exome sequencing. One would expect the finding of recurrent variants in the same gene in at least two independent tumor samples to support a role in pathogenesis. If mutations in an unknown gene are present in 5 % of the samples, the binomial probability of finding mutations in at least two samples (assuming perfect sensitivity of the assay) is about 96 % when 100 samples are sequenced. This likelihood drops to 72 % with 50 samples sequenced, and 36 % with 25 samples sequenced. These considerations suggest that a meta-analysis of exome sequencing studies from different groups may reveal additional genes involved in APA pathogenesis.

Third, copy number variation (CNV) has been described in APAs [11], but its role in the pathophysiology of APAs remains unclear. CNV may well account for a fraction of APAs, and the identification of one or several overlapping areas of CNV between tumors may point to key pathways involved in aldosterone production and/or proliferation. Another possibility is the co-occurrence of predisposing single-nucleotide variants (SNVs) in the germline and CNV in corresponding tumor samples – adrenals with several APAs and/or accompanying hyperplasia might be good candidates for such a disease mechanism.
Another open question is the role of CTNNB1 mutations in APAs. It is stunning that CTNNB1 variants are found in non-producing adrenal adenomas, adrenocortical carcinomas, cortisol-producing adenomas as well as APAs [19–21, 30–34]. Some cortisol-producing adenomas with CTNNB1 variants show additional GNAS variants, which explain hormone production [34, 35], but so far, no mutations in second genes have been described in APAs with CTNNB1 variants. One group suggested an association of APAs with CTNNB1 mutations with pregnancy, presumably due to expression and activation of gonadal receptors [36], but this seems highly unlikely because about 25% of CTNNB1-positive tumors occur in males, and because these variants are more common in post-menopausal females than in pregnant women [20, 21, 37]. Similarly, expression of gonadal receptors as well as CYP11B2 was subsequently shown to be heterogeneous [21]. Taken together, these findings suggest that CTNNB1 variants may cause adrenal proliferation by activating Wnt signaling, but not excessive hormone production. The factors driving aldosterone production remain to be discovered.

Lastly, open questions concern the origin of KCNJ5-positive tumors. It has been suggested early on and confirmed that these tumors are histologically different from tumors with mutations in other genes; they tend to have a more fasciculata-like appearance [16, 19, 38]. It is therefore interesting that KCNJ5 variants are not found in APCCs [22]. Do KCNJ5-positive tumors arise from zona fasciculata [38]? Do they grow so fast that precursors evade detection as APCCs? Related to this question, it is unclear why KCNJ5 mutations are more prevalent in females than in males in European and some Asian cohorts [12]. A higher mutation rate seems unlikely given that such female preponderance is absent in those with other mutations [13, 17]. An effect of gonadal steroids on proliferation or hormone production may play a role.

Another area of "known unknowns" is the genetic basis of FH. There are a number of unsolved kindreds and/or cases. These include an extended kindred with so-called FH-II (non-glucocorticoid remediable aldosteronism) described in 1992 [39, 40], but also many additional cases of childhood-onset PA. In one analysis, a CACNA1H variant was identified in five of 40 unrelated subjects with hypertension due to PA by age 10 years [28], yet the remaining cases are unsolved. Whereas there is no proof that aldosteronism in these cases is Mendelian, the index of suspicion is high. APAs are increasingly rare in the pediatric age range, as is sporadic bilateral adrenal hyperplasia. These observations suggest that there are likely additional genes to be discovered in FH. Incomplete penetrance, small kindreds and the presence of phenocopies (subjects with sporadic PA in the family) are potential challenges in the analysis.

The Unknown Unknowns

The major "unknown unknown" is a potential genetic basis of sporadic BAH, also known as idiopathic hyperaldosteronism. Patients with BAH may show nodular changes on adrenal CT imaging, or CT
may be normal [4]. Because these patients typically do not undergo surgery, BAH is a histologically ill-defined entity. If the diagnosis is based on adrenal venous sampling showing bilateral aldosterone production [4], potential correlations may include bilateral microscopics hyperplasia, bilateral nodular hyperplasia, bilateral adenomas [41, 42], bilateral APCCs or any combination of these entities.

Thus, genetic contributions could include somatic variants in adenomas or APCCs, or predisposing germline variants. Non-genetic causes may include hypothetical serum factors or auto-antibodies against the angiotensin II type 1 receptor [43].

How could potential genetic contributors be studied? One approach would be to perform next-generation sequencing of lesions in the rare cases that have undergone surgery and search for somatic variants [42]. This may include cases operated based on presumed lateralization without cure [44]. Another option would be to study adrenals from autopsies – perhaps patients with known BAH could be part of a study that involves post-mortem removal of adrenal glands, or autopsy cases with BAH positive for aldosterone synthase expression could be studied. A case report suggests that elevated aldosterone levels can be determined from postmortem blood [45], although reference ranges would have to be determined. Post mortem urinary aldosterone may also prove useful. Perhaps assessment of the juxtaglomerular apparatus could serve to distinguish primary from secondary aldosteronism [46]. Regarding potential germline variants, whether genome-wide association studies (GWAS) or next-generation sequencing studies would be helpful remains to be determined. Prior GWAS studies have focused on aldosterone to renin ratio in a population-based cohort (not PA) [47] or candidate gene association with aldosterone levels [48].

**Outlook**

The expected progress in solving unanswered questions in the genetic basis of PA over the next few years seems straightforward regarding the "known unknowns". We will likely see the discovery of genetic variants in one or several additional genes that explain a small fraction of APAs, and we will likely see a better characterization of CNVs in APAs. This is expected to result in new insight into the pathophysiology of APAs and PA. We should also anticipate the discovery of variants in (a) new gene(s) in FH, with similar pathophysiological implications. Future animal models may help to better understand the development of KCNJ5-mutant tumors.

Much more difficult to predict is the progress on the genetic basis of bilateral adrenal hyperplasia, although a contribution of somatic variants seems possible. In any case, the exciting times of genetic discoveries in PA will likely continue.

**Acknowledgements**

U.I.S. is supported by grants of the Ministerium für Kultur und Wissenschaft des Landes Nordrhein-Westfalen, Germany (NRW-Rückkehrprogramm, Junges Kolleg), the Deutsche Forschungsgemeinschaft (SCHO 1386/2-1) and the Fritz Thyssen Stiftung (10.16.1.027MN).

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**Conflict of Interest**

The author is listed as an inventor on a patent application by Yale University (Compositions and Methods for Diagnosing and Treating Diseases and Disorders Associated with Mutant KCNJ5).


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