Primary aldosteronism diagnostics: KCNJ5 mutations and hybrid steroid synthesis in aldosterone-producing adenomas

Juilee Rege¹, Adina F. Turcu², William E. Rainey¹²

¹Department of Molecular and Integrative Physiology, ²Division of Metabolism, Endocrinology, and Diabetes, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA

Contributions: (I) Conception and design: J Rege, WE Rainey; (II) Administrative support: WE Rainey; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: J Rege, WE Rainey; (V) Data analysis and interpretation: None; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: William E. Rainey, PhD. Departments of Molecular and Integrative Physiology and Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA. Email: WER@umich.edu.

Abstract: Primary aldosteronism (PA) is characterized by autonomous aldosterone production by renin-independent mechanisms and is most commonly sporadic. While 60–70% of sporadic PA can be attributed to bilateral hyperaldosteronism, the remaining 30–40% is caused by a unilateral aldosterone-producing adenoma (APA). Somatic mutations in or near the selectivity filter the KCNJ5 gene (encoding the potassium channel GIRK4) have been implicated in the pathogenesis of both sporadic and familial PA. Several studies using tumor tissue, peripheral and adrenal vein samples from PA patients have demonstrated that along with aldosterone, the hybrid steroids 18-hydroxycortisol (18OHF) and 18-oxocortisol (18oxoF) are a hallmark of APA harboring KCNJ5 mutations. Herein, we review the recent advances with respect to the molecular mechanisms underlying the pathogenesis of PA and the steroidogenic fingerprints of KCNJ5 mutations. In addition, we present an outlook toward the future of PA subtyping and diagnostic work-up utilizing steroid profiling.

Keywords: Aldosterone-producing adenomas; KCNJ5; CYP11B2; CYP17A1; 18-hydroxycortisol; 18-oxocortisol; aldosterone

Submitted Oct 22, 2019. Accepted for publication Nov 06, 2019.
doi: 10.21037/gs.2019.10.22
View this article at: http://dx.doi.org/10.21037/gs.2019.10.22

Introduction

Primary aldosteronism (PA) is the most common form of secondary hypertension, and it accounts for 5–8% of hypertension (1-6) and 11–20% of resistant hypertension (7-9). PA is characterized by inappropiate autonomous production of aldosterone via renin-independent mechanisms (10). Most PA patients exhibit a sporadic form, whereas 5–6% of the cases are caused by familial disease (11). Approximately 60–70% of PA cases can be attributed to bilateral hyperaldosteronism (BHA), with the remaining 30–40% being caused by unilateral aldosterone-producing adenomas (APA) (2,12). Uncommon forms of PA include unilateral adrenal hyperplasia and adrenal carcinoma (13).

Differentiation of the uni- and bilateral forms of PA is important for guiding therapy. Unilateral PA can benefit from adrenalectomy and BHA requires indefinite medical therapy, which typically incorporates a mineralocorticoid receptor antagonists (MRA) (14,15). Adrenal vein sampling (AVS) is the most reliable method for distinguishing between APA and BHA (10). However, there are several caveats to the AVS methodology and interpretation of hormonal data. Despite being highly predictive of outcome (10), this procedure is laborious, invasive, and expensive. Additionally, AVS is performed in a limited number of referral centers, and it is dependent on highly-skilled interventional radiologists with large annual AVS volume (16-18).
A simple blood test that identifies APA-derived serum steroid biomarkers would help distinguish patients who will benefit from adrenalectomy from those who should be treated with medical therapy. Such biomarkers could also conserve healthcare resources by sparing many patients expensive imaging and invasive studies. Finally, the utility of such biomarkers would increase the rate of PA screening, facilitate PA diagnosis and appropriate treatment, and thus reduce the burden of cardiovascular and renal complications which affect PA patients disproportionately more than those with essential hypertension.

**Hybrid steroids as diagnostic markers for differentiation of APA from BHA**

Over the past 25 years, the applicability of the 18-oxygenated derivatives of cortisol, 18-oxocortisol (18oxoF) and 18-hydroxycortisol (18OHF) for subtyping PA as uni- or bilateral, has been of interest (19-27). Several studies have shown higher levels of 18OHF and 18oxoF in PA patients compared to those with essential hypertension (23,28-30). High concentrations were also demonstrated in patients with familial hyperaldosteronism type 1 (FH type I) (31,32). FH type I accounts for <1% of cases of PA (11) and is caused by a chimeric gene that is composed of the promoter of 11β-hydroxylase (CYP11B1) fused with the coding region of aldosterone synthase (CYP11B2) (31). Like CYP11B1, this CYP11B1/CYP11B2 chimeric enzyme is present in the zona fasciculata (ZF) of the adrenal cortex and is regulated by ACTH. As a result, the CYP11B1/CYP11B2 chimera is able to use cortisol as a substrate to produce 18OHF, which is further metabolized to 18oxoF (19,23,30,32-34) (Figure 1). These metabolites of cortisol are designated as “hybrid” steroids owing to their molecular structure comprising features of steroid metabolism which typically occur in the zona glomerulosa (ZG) (18-hydroxylation and 18-oxidation) and the ZF (17-hydroxylation) (31) (Figure 1).

In the normal adrenal gland, expression of CYP11B2 is restricted to the ZG, while 17α-hydroxylase/17,20-lyase (CYP17A1) and CYP11B1 are expressed exclusively in the ZF and zona reticularis (Figure 2), thereby leading to low production of 18OHF and 18oxoF in normal subjects. Histologic studies have shown that some APA display a ZG-
like phenotype, with small, compact cells, while other APA are composed of large, lipid-rich cells, similar to those seen in ZF (35-38). Although transcriptomic analysis has not detected any differences in \textit{CYP11B2} mRNA expression between the different APA subtypes (39), higher \textit{CYP11B2} protein expression has been observed in the APA with ZG-like cells in some studies (35,37). Specific to APA with ZF-like histology is a higher expression of steroidogenic enzymes required for cortisol biosynthesis, such as \textit{CYP17A1} and \textit{CYP11B1} (40-42). The co-expression of \textit{CYP17A1} and \textit{CYP11B2} in these APA facilitates the production of hybrid steroids (21,33,43) (Figures 1,2).

Ulick et al. performed the initial PA hybrid steroid studies in 1993 and demonstrated that urinary 18OHF and 18oxoF were elevated in patients with APA compared to those with BHA (21,22). Numerous immunoassay studies followed that indicated higher plasma and urinary 18OHF and 18oxoF levels in subjects with APA vs. those with BHA (19,23,29). More recently liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to quantify the hybrid steroids in APA and BHA, confirming some of the previous immunoassay analyses (20,25). Satoh et al. measured 18OHF and 18oxoF in the peripheral plasma of 234 Japanese PA patients by LC-MS/MS and found that these steroids could discriminate APA from BHA with considerable specificity and sensitivity (20). In contrast, a European study of 216 PA patients indicated that both 18oxoF and 18OHF displayed significant overlap between APA and BHA, thereby suggesting a limitation in the utility of these steroids as discriminators between the two PA subtypes (24). Nevertheless, this analysis presented a composite of 12 steroids that was able to correctly classify the PA subtype in 80% of the patients (24). A subsequent study from the same group revealed that APA with different underlying somatic mutations produce specific steroid fingerprints (44).

**The hybrid steroids and KCNJ5 somatic mutation connection in APA**

In 2016, Williams et al. identified specific steroid fingerprints in adrenal vein (AV) and peripheral vein (PV) plasma from patients with APA with various underlying mutations (44). Of the 79 PA patients with unilateral PA included, 34% had APA harboring \textit{KCNJ5} (encoding the G protein-coupled inward-rectifying potassium channel 4, GIRK4) mutations, 11% had ATPase (\textit{ATP1A1} (\textit{Na}^+/K^+ ATPase \alpha1-subunit)), \textit{ATP2B3} (\textit{Ca}^{2+} ATPase 3) mutations and 9% had \textit{CACNA1D} (encoding the voltage-dependent L-type calcium channel subunit \alpha1-D, Cav1.3) mutations. In the remaining 46% of APA no mutations were identified. Patients with \textit{KCNJ5}-harboring APA had the highest concentrations of 18OHF and 18oxoF in both the AV and PV plasma (44). The elevated levels of the hybrid steroids produced by the \textit{KCNJ5}-mutated APA could be explained by their predominantly ZF phenotype (elevated \textit{CYP17A1}) along with \textit{CYP11B2} expression (35-38) (Figure 2). Conversely, APA with \textit{ATP1A1}, \textit{ATP2B3}, and \textit{CACNA1D} mutations, were shown to be
smaller in size and to be composed principally of ZG-like cells (35-38). Assembling a 7-steroid panel measured in PV plasma, including aldosterone, 18oxoF, 18OHF, 11-deoxy cortisol (11-DOC), cortisol, androstenedione, and 21-deoxycortisol, William et al. were able to classify 92% of APA according to the underlying somatic mutation (44). In a subsequent study, the same group used steroid profiling to differentiate patients with micro-APA, macro-APA and BHA (45). Patients with macro-APAs, which frequently harbor KCNJ5 mutations, displayed higher concentrations of aldosterone and the hybrid steroids as compared with patients with micro-APA and BHA (45). These findings were in concordance with a recent analysis of APA tissue by comprehensive mass spectrometry imaging in relation to mutation status, immunohistochemical reports of steroidogenic enzymes and steroid profiles from 139 patients (46). Increased intratumoral intensities of 18OHF and 18oxoF were seen in KCNJ5-mutated APA. Additionally, two in vitro studies corroborated the finding that expression of a KCNJ5 mutation in the adrenocortical HAC15 cell line results in a significant increase in CYP11B2 gene transcription, and elevation in the production of aldosterone and the hybrid steroids (47,48).

The utility of hybrid steroids as promising discriminators between APA and BHA in Japanese patients with PA (20) could be attributed to the high prevalence of APA KCNJ5 mutations in this population (49,50). Tezuka et al. recently highlighted the potential of 18oxoF as biomarker for KCNJ5-harboring APA in Japanese patients in a study that measured its intratumoral and peripheral serum levels in patients with PA who underwent unilateral adrenalectomy (51). This study showed that APA harboring KCNJ5 mutations demonstrated enhanced synthesis of 18oxoF owing to elevated intratumoral cortisol production which could be used as substrate by CYP11B2. These tumors also had increased CYP11B1 and CYP11B2 double-positive hybrid cells compared with APA harboring the wild-type KCNJ5 gene (51). In addition to aberrant KCNJ5-related sporadic PA, the hybrid steroids have also been shown to be elevated in familial hyperaldosteronism type III (FH type III) (52,53) which was described by Geller et al. in 2008 as an early onset and severe form of primary aldosteronism (52) and was shown to be caused by germline mutations in KCNJ5 (53).

**Somatic KCNJ5 mutations in APA—the mechanics**

The product of KCNJ5, GIRK4 is a member of the G protein-activated inwardly rectifying K+ channel subfamily and is localized on the plasma membrane of tissues such as the heart, central and peripheral neurons along with various endocrine tissues (54,55). Tissue transcriptome analysis, however, suggests that the adrenal is by far the tissue with highest levels of the transcript encoding GIRK4. ‘Inward rectifiers’ are a class of K+ channels that conduct large currents in the inward direction at membrane voltages negative to the K+ equilibrium potential. The primary structure of this channel consists of 2 membrane spanning helices flanking one extracellular pore-forming region in between and cytoplasmic N- and C-termini that contribute to the pore structure (56,57). The pore-forming domain constitutes the K+ ion selectivity filter of the channel which is characterized by the signature sequence Gly-Tyr-Gly (57,58). This sequence allows stringent passage of the larger K+ ions through the channel into the cell and prevents the entry of smaller, more abundant Na+ ions (59). Immunohistochemical studies have shown that GIRK4 is localized mainly to the ZG of the human adrenal cortex and to the outer part of the ZF (40,53,60). GIRK4 and other K+ channels maintain the hyperpolarized state of the ZG cell by allowing an outward flow of K+ conductance (54,55) (Figure 3).

The advent of large-scale methods of analyses such as gene sequencing [e.g., next generation sequencing (NGS) and whole exome sequencing] in the last decade has helped to elucidate the genetic landscape and molecular mechanism of PA pathogenesis. The genetic basis of PA was largely an uncharted territory until Choi et al. used exome sequencing to first report a role for somatic mutations in driving autonomous aldosterone production in APA in 2011 (53). This study identified two “hot-spot” somatic mutations (p.Gly151Arg (G151R) and p.Leu168Arg (L168R) substitutions) in KCNJ5 (53) (Figure 4). These originally described KCNJ5 somatic gain-of-function mutations were located near or within the selectivity filter and disrupt its ion selectivity by facilitating indiscriminate entry of Na+ through the pore of the outer tunnel (53). The resulting depolarization of the cell membrane induces the opening of the voltage-gated Ca2+ channels leading to elevated intracellular Ca2+, increased activation of the calcium signaling pathway, augmented CYP11B2 transcription and aldosterone biosynthesis (Figure 3). Two cell-based analyses from our group established that mutated KCNJ5 activates the acute and chronic regulatory steps of aldosterone production and that increased aldosterone production occurs along with elevations in CYP11B2, and...
its regulatory transcription factors nuclear receptor related 1 protein (NURR1) and activating transcription factor 2 (ATF2) (47,60). Besides G151R and L168R which constitute 90% of all the KCNJ5 APA mutations, 24 other KCNJ5 mutations have also been detected in APA (40,41,53,61-70,74-77) (Figure 4).

Along with their studies on the genetic causes of somatic APA, Choi et al. also established the genetic basis of FH type III by identifying a novel gain-of-function KCNJ5 germline mutation in a father and his two daughters, all with PA (53). This substitution mutation—p.Thr158Ala (T158A)—is located near the selectivity filter of the channel pore. The

---

**Figure 3** Regulation of adrenal aldosterone production by wild type and mutated GIRK4 (KCNJ5). (A) Adrenal glomerulosa cells with wild type GIRK4 (KCNJ5) are in a hyperpolarized state as a result of high resting K⁺ conductance. (B) In pathological conditions, adrenal cells carrying mutations in GIRK4 (KCNJ5) demonstrate indiscriminate conductance of Na⁺, resulting in chronic cell membrane depolarization and constitutive activation of CYP11B2 and aldosterone production.

**Figure 4** KCNJ5 mutations in APA. The most common KCNJ5 mutations (G151R, L168R and T158A) occur in or near the selectivity filter (38,43,44,56,64-66,68-77). Other reported mutations are listed in the table.
T158A mutation was later also detected in sporadic APA by Mulatero et al. in 2012 (76). Adrenocortical cell line studies demonstrated that the p.Thr158Ala mutation in KCNJ5 causes an increase in aldosterone production via membrane depolarization and Na⁺ and Ca²⁺ influx (47,48). While patients carrying the germline mutations G151R (similar to the recurrent somatic KCNJ5 mutation in APA), T158A and p.Ile157Ser, presented with early onset and a severe PA phenotype with drug-resistant hypertension and adrenal hyperplasia (71,72), those with the p.Gly151Glu (G151E) (72) and p.Tyr152Cys (73) variants exhibited a remarkably milder phenotype.

Somatic KCNJ5 mutations in APA—the demographics

The last 10 years have resulted in a plethora of studies in the field of PA that have investigated the presence and racial prevalence of the various aldosterone-driving somatic mutations in APA, including KCNJ5. Collaborating investigators from the European Network for the Study of Adrenal Tumors (ENS@T) have conducted the largest mutation prevalence studies to date assessing 474 APA with the sequencing directed at the previously reported mutation hotspots (39). This multicenter study demonstrated the presence of somatic mutations in 54% of APA, with genetic abnormalities in KCNJ5 representing 38% (39). This study corroborated the frequency of somatic mutations in KCNJ5 that were identified in previous smaller studies in European populations (35,74,75,78,79). Of note, the prevalence of reported somatic mutations in APA has been shown to vary by race. In particular, somatic KCNJ5 mutations are much more common in East Asian patients than in Europeans (70% vs. 38%) (36,49,50,65,68,80-82,85). The association of KCNJ5 mutations with sex (women higher than men), younger age, pronounced hyperaldosteronism and larger tumor size was also demonstrated in a meta-analysis study comprising 1,636 APA patients from 13 studies (86).

Conclusions and perspectives

The current diagnostic work-up for PA is a complex multitiered process (10). Although the Endocrine Society Guidelines recommend measurement of the aldosterone-to-renin ratio (ARR) as an appropriate initial screening test for PA, ARR is only about 80% specific and sensitive (87-89). Furthermore, it requires confirmatory testing of aldosterone production following sodium loading and volume expansion (10). More importantly, the ARR is elevated in patients with both BHA and APA. Consequently, the biochemical diagnosis of PA does not differentiate between the two primary causes of PA, and follow-up procedures are needed to classify patients with PA. The major impediments to screening for and treating PA are the complexities in the later stages of the evaluation. Currently available laboratory tests can confirm the diagnosis of PA, but neither these tests, nor current imaging studies can determine which adrenal gland(s) is (are) the source(s) of aldosterone. There is general agreement that cross-sectional imaging studies such as computed tomography scanning or magnetic resonance imaging cannot distinguish between most APA and BHA cases. This failure derives from both the small size of most APA and the high prevalence of nonfunctional adrenocortical adenomas in
the general population, most of which do not produce aldosterone. Thus, the expensive, technically cumbersome and invasive AVS technique remains the gold standard for the subtyping of patients with PA. The variable success rates of AVS methodology from center-to-center has prompted researchers to consider alternative non-invasive diagnostic tools that could aid in diagnosing and classifying the different forms of PA.

Recent advances in the diagnostics of PA have been possible with the emergence of the LC-MS/MS methodology which allows steroid profiling in an individual patient. Increasing evidence suggests that steroid fingerprinting might be a major determinant in not only differentiating APA from BHA, but also in providing genotype-phenotype associations of APA, thereby making it a useful tool in simplifying the complex diagnostic work-up in patients with suspected PA. The utility of hybrid steroids—18OHF and 18oxoF—as potential differentiators between APA and BHA, has been tested for the past three decades with encouraging results. In fact, a panel of 12 steroids including the hybrid steroids in peripheral plasma that was put forth by Eisenhofer et al. was successful in classifying 80% of the PA cases as APA or BHA (24). The same research group also demonstrated that the putative application of steroid profiling for subtype classification of PA is likely due to the association of the steroid metabolome with somatic APA aldosterone-driver mutations (44). Notably, they showed that the high levels of hybrid steroids could be a signature for KCNJ5-mutated APA. Moreover, steroid profiling of peripheral blood was able to correctly categorize 92% of the somatic APA mutations. They recently took a similar diagnostic route in the case of a 55-year-old female patient with left adrenal mass in whom AVS was a failure (90). The patient's peripheral plasma displayed increased levels of aldosterone, 18OHF, 18oxoF, 11-DOC, 11-deoxycortisol (90) which were indicative of a macro-APA as a result of a KCNJ5 mutation (45). Adrenalectomy was recommended for this patient and the diagnosis was later confirmed by genetic testing and histopathology (90). This example highlights the clinical applicability of steroid fingerprinting in the PA work-up.

The inclusion of steroid profiling in the PA diagnostic chart has several advantages. Sixty percent to 70% of patients with PA have BHA and thus receive no benefit from the AVS procedure, because they are treated medically rather than surgically. Certain populations would benefit from the new diagnostic approach following ARR wherein steroid profiling could help identify patients that need to undergo the CT and AVS and bypass invasive testing in patients with BHA, who require lifelong personalized medical treatment. KCNJ5 mutations, which are more prevalent in women and constitute the majority of East Asian APA cases of both sexes, could be diagnosed rapidly by serum hybrid steroids. Given the high prevalence of KCNJ5 mutations in PA patients, macrolides might potentially be used to identify patients that bear APA with KCNJ5 mutations (91) and as targeted personalized treatments for these patients. Inclusion of steroid fingerprinting in the PA diagnostic work-up will reduce healthcare cost and increase the impact on patient safety for almost 60–70% PA patients by reducing radiation exposure and application of invasive procedures. Prospective studies will, however, be needed to validate whether this method is a better alternative to the invasive and technically onerous AVS.

Acknowledgments

Funding: This work was supported by grants K08 DK109116 A.F.T and R01 DK106618 and R01 DK043140 to W.E.R. from the National Institutes of Health (NIH), and grant 2019087 from the Doris Duke Charitable Foundation to AFT.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Open Access Statement: This is an Open Access article distributed in accordance with the Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International License (CC BY-NC-ND 4.0), which permits the non-commercial replication and distribution of the article with the strict proviso that no changes or edits are made and the original work is properly cited (including links to both the formal publication through the relevant DOI and the license). See: https://creativecommons.org/licenses/by-nc-nd/4.0/.
References


78. Beuschlein F, Bouklroun S, Osswald A, et al. Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-


