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Aldosterone-producing cell clusters in normal and pathological states

Kei Omata^{1,7,8}, Scott A. Tomlins^{1,2,3,4}, and William E. Rainey^{5,6}

¹Department of Pathology, University of Michigan, 1500 E Medical Center Drive, 48109 Ann Arbor, MI, USA

²Michigan Center for Translational Pathology, University of Michigan, 1500 E Medical Center Drive, 48109 Ann Arbor, MI, USA

³Department of Urology, University of Michigan, 1500 E Medical Center Drive, 48109 Ann Arbor, MI, USA

⁴Comprehensive Cancer Center, University of Michigan, 1500 E Medical Center Drive, 48109 Ann Arbor, MI, USA

⁵Department of Molecular and Integrative Physiology, University of Michigan, 1500 E Medical Center Drive, 48109 Ann Arbor, MI, USA

⁶Department of Medicine, University of Michigan, 1500 E Medical Center Drive, 48109 Ann Arbor, MI, USA

⁷Division of Nephrology, Endocrinology and Vascular Medicine, Tohoku University, 2-1 Seiryomachi, Aoba-ku, 980-0872 Sendai, Miyagi, Japan

⁸Division of Clinical Hypertension, Endocrinology & Metabolism, Tohoku University, 2-1 Seiryomachi, Aoba-ku, 980-0872 Sendai, Miyagi, Japan

Abstract

Primary aldosteronism (PA) plays an important role for the risk of cardiovascular complications, and early diagnosis and targeted treatment in the early states based on its pathophysiology is warranted. Next-generation sequencing (NGS) has revealed recurrent somatic mutations in aldosterone-driving genes in aldosterone-producing adenoma (APA). By applying CYP11B2 (aldosterone synthase) immunohistochemistry and NGS to adrenal glands from normal subjects and PA patients, we and others have shown that CYP11B2-positive cells make small clusters, termed aldosterone-producing cell clusters (APCC), beneath adrenal capsule and harbor somatic mutations in genes mutated in APA. We have shown that APCC are increased in CT-negative PA adrenals, while others showed potential progression from APCC to micro APA through mutations.

CORRESPONDING AUTHORS: William E. Rainey, Ph. D., Jerome W. Conn Professor, Molecular & Integrative Physiology and Internal Medicine, University of Michigan, 2560D, MSRB II, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-5622, Phone: +1-734-764-7514, wer@umich.edu.

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These results suggest that APCC are a key factor for understanding the origin of PA, and further investigation on the relation between APCC and PA is highly needed.

Keywords

Primary aldosteronism; aldosterone-producing cell clusters; next -generation sequencing

1. Primary aldosteronism and genetic causes of aldosterone-producing adenoma (APA)

Primary aldosteronism (PA) is caused by overproduction of aldosterone in one or both adrenal glands, and is clinically characterized by high aldosterone levels despite suppressed renin. Diagnosis and targeted treatment in the early stages of this disease is important because it affects about 6–10% of hypertensive patients (high prevalence) and the risk for cardiovascular complications is significantly higher than is seen in age-, sex-, and blood pressure-matched essential hypertension (increased cardiovascular risk)[1,2].

Defining the most appropriate therapy for PA is heavily influenced by the laterality of aldosterone overproduction as determined by adrenal vein sampling (AVS). When aldosterone is overproduced in one adrenal gland (mostly by CT-scan detectable APA or CT-undetectable unilateral multiple adrenocortical nodules [UMN][3]), unilateral adrenalectomy is recommended as a likely cure for hyperaldosteronism[1]. In contrast, mineralocorticoid receptor antagonists are used when AVS indicates bilateral hyperaldosteronism (BHA). BHA can be further subdivided into CT-negative idiopathic hyperaldosteronism (IHA) and rarely CT-positive bilateral APA[4,5]. Although there continues to be a broadening recognition of PA as a major contributor to cardiovascular diseases, defining the cellular and genetic causes of PA remains an area for active research. Defining the origins and causes of PA could further improve our current methods for early detection and provide more efficient targeted therapies.

Recently, next-generation sequencing (NGS) revealed recurrent somatic mutations in APA[6–9]. *KCNJ5*, potassium voltage-gated channel subfamily J member 5, is the most commonly mutated gene, accounting for approximately 40% and 70% of APA in Caucasian and Eastern Asian patients[8,10–16] (Figure 1). In addition, *CACNA1D*, *ATP1A1* and *ATP2B3* (calcium voltage-gated channel subunit alpha1 D, ATPase Na+/K+ transporting subunit alpha 1, ATPase plasma membrane Ca²⁺ transporting 3, respectively) each account for up to 10% of APA, for Caucasians and Eastern Asian PA patients[8,10–16]. Mechanistically, mutations in these genes appear to converge on an elevation of intracellular calcium in adrenal cells. This increase in calcium is believed to cause CYP11B2 expression and aldosterone overproduction[6–9,17,18]. *CTNNB1* mutations are also reported in APA and its involvement in tumorigenesis and aldosterone overproduction is an active field of research.

2. Early descriptions of aldosterone-producing cell clusters (APCC)

It has long been known that aldosterone-producing cells express the enzyme aldosterone synthase (CYP11B2). This enzyme carries out the final steps in aldosterone synthesis and, under normal situations, is not expressed outside of the adrenal zona glomerulosa[19]. In 2010, Nishimoto et al reported a population of CYP11B2-expressing cells that appeared as clusters beneath the adrenal capsule, and termed them APCC[20]. These cells were distinct from the normal zona glomerulosa cells in that APCC extended into the zona fasciculata. Boulkroun et al also observed CYP11B2-positive structures in subcapsular regions and using IHC for the glomerulosa marker, Dab2, were able to distinguish three structures that they termed foci, megafoci and APCC[21]. The relative role of each of these structures in physiologic vs inappropriate aldosterone production warrants further research. Interestingly, APCC were also present in surgically removed PA adrenal glands adjacent to an APA[20,21], suggesting that APCC had autonomous CYP11B2 and aldosterone production in the presence of the systemically suppressed renin-angiotensin-aldosterone system in PA patients.

In 2015, we further showed APCC are present in adrenals from kidney donors and demonstrated that 6/23 (26%) of APCC harbor somatic mutations, predominantly in *CACNA1D* (but did not observe *KCNJ5* mutations) (Figure 1)[22]. Transcriptome analysis of APCC demonstrated that they were more similar to the adrenal zona glomerulosa than zona fasciculata or reticularis cells. The demonstration of somatic mutations in APCC further suggested a potential role of these cells in the dysregulation of aldosterone production.

3. APCC to CT-negative PA pathway

Next, we have used adrenal glands from unilateral CT-negative PA (UMN) patients for CYP11B2 immunohistochemistry (IHC) and targeted NGS[23]. By IHC, we observed adrenals with multiple CYP11B2-positive nodules but without identifiable adenomas, potentially suggesting these micronodules were the source of excess aldosterone in these PA patients. Interestingly, the NGS analysis showed that these micronodules had an APCC-like mutation spectrum (*CACNA1D* >> *KCNJ5*), similar to the previous cohort of APCC from Caucasian kidney donors[22].

This study led to the hypothesis that the increased number of adrenal CYP11B2-expressing nodules in this group of CT-negative PA patients was the cause of hyperaldosteronism. To test this hypothesis, we then collected a large cohort of Japanese normotensive adrenal glands and compared the APCC number with that of the UMN cohort described above[24]. Importantly, the APCC number of the CT-negative PA adrenals was significantly higher than that of normotensive adrenals, supporting a role for APCC in the pathologic progression and clinical manifestation of hyperaldosteronism in CT-negative PA. Moreover, the somatic mutation spectrum of APCC observed in this normotensive cohort (predominantly in *CACNA1D*) was similar to that of CT-undetectable PA adrenals (Figure 1)[23], implying that normal adrenal glands may progress to a CT-undetectable PA through an increase in APCC number. However, APCC appear unlikely to be the precursors to APA in CT-

detectable cases where mutations in *KCNJ5* are the major causative alterations (Figure 1). Morphology and IHC further support this pathway. In both UMN and normotensive cohorts, we showed that many APCC were composed of morphologically normal ZG and ZF cells without adenomatous characteristics frequently seen in APA (such as a fibrous capsule, intratumoral heterogeneity of clear and compact cortical cells, and/or the occasionally detected tumor cell atypia with enlarged nuclei). We have also shown that APCC in both cohorts were generally negative for CYP11B1 and CYP17A1, enzymes responsible for cortisol production, in contrast to APA where CYP11B1 and CYP17A IHC are generally positive[23,24] (Figure 2).

We also showed that APCC accumulate with age in normal adrenals, and CT-negative PA could be considered a pathologic variant for age-associated APCC expansion. The age-associated increase in adrenal APCC was confirmed in an independent cohort of Japanese autopsy cases without defined hypertensive status[25] and American renal-donor derived adrenals[26]. The potential for an age-associated APCC-driven disturbance of aldosterone production that could represent pre-clinical forms of PA has been suggested but further work will be needed to establish such a role.

Importantly, a recent study showed that excess aldosterone caused by *CACNA1D* mutations could be inhibited by calcium channel blockers[27], suggesting that such drugs might be useful for APCC-oriented aldosterone overproduction. The current findings support a contribution of APCC to unilateral CT-negative PA, but an APCC role in the more common bilateral CT-negative PA (IHA) remains to be determined.

4. APCC to APA pathway

Nishimoto et al originally proposed the concept that APCC might be the precursor of APA since APCC produce aldosterone autonomously and harbor somatic mutations observed in APA[22]. Following this report, they described several UMN cases in which CYP11B2-positive regions harbor both inner micro-APA-like and subcapsular APCC-like components[28]. These regions were termed possible APCC-to-APA transitional lesions (pAATLs), some of which harbored aldosterone-driver mutations only in micro-APA-like portions but not in APCC-like portions. The authors suggest that micro-APA may arise from existing mutation-negative APCC through acquisition of APA-related mutations.

5. Definition of APCC

As reviewed above, APCC are reported to produce aldosterone autonomously through aldosterone-stimulating mutations and CYP11B2 overexpression, and believed to play a key role in PA development. However, a clear definition of APCC that can differentiate this unique structure from small APA and adjacent normal ZG cells producing physiological amount of aldosterone (without mutations) is not yet established. Some studies have used CYP11B2 IHC and its size to detect APCC[20,22], while others incorporated Dab2 IHC in addition to CYP11B2[21].

In our observations, APCC did not harbor structural features frequently seen in APA, such as a capsule and cellular/tissue atypia. CYP11B1 and CYP17A1 IHC were also revealed to be

useful to differentiate APCC from APA as CYP11B1 and CYP17A1 IHC were negative in APCC, which are generally positive in APA[23,24]. However, whether APCC and small APA can be (or should be) clearly distinguished awaits future study.

To distinguish APCC from normal adrenal cells may be more difficult as the cells composing APCC and their alignment appear to be extremely similar to those of non-APCC[23,24]. Of particular interest, ZF-like cells in APCC are generally positive for CYP11B2 IHC[23,24]. This may help to identify APCC since ZF cells in physiological conditions are reported to be negative for CYP11B2 IHC and unrelated to aldosterone production[19,29]. NGS may also help to distinguish APCC from adjacent normal cells because the observed variant allele frequency in APCC supports that the mutation is present in both the ZG and ZF-like components in APCC and absent in surrounding cells negative for CYP11B2[24]. This result suggests a clonal nature for the alterations in APCC and may explain the pathological IHC status of CYP11B2 in ZF cells in APCC.

6. Potential of IHC-guided NGS

NGS for APCC relies on the accurate detection and isolation of APCC. Recent development of more selective IHC CYP11B2 antibodies[19,29] has allowed improved visualization of APCC. NGS from APCCs is technically challenging due to the need to capture relatively pure cell populations and the limited amount of isolatable DNA. Importantly, the small size of APCC (the average size is 0.15 mm²)[24] is coupled with the usual method of identifying them on formalin-fixed paraffin-embedded (FFPE) specimens, which typically produce lower quality DNA than fresh frozen material. Hence, optimized methodologies, as developed by us and others, are required for accurate NGS from APCC.

We developed a small targeted NGS panel (APAv1) targeting the complete coding sequence of several known aldosterone-driver genes discovered as recurrently mutated in APA[22]. Using this approach, each nucleotide position can be covered by hundreds to thousands of reads, enabling detection of even low frequency mutations even with limited DNA from FFPE APCC samples. Importantly, aldosterone regulating gene mutations are largely activating (and hence at recurrently altered positions), enabling more easy variant detection than discovery based sequencing or the need to identify deleterious mutations across a gene of interest. We also demonstrated the ability to profile APCCs using frozen materials (OCT processed adrenals) using laser-capture microcopy to improve the DNA/RNA sample quality. Unfortunately, LCM from fresh material requires prospective procurement of samples, and is time consuming in routine practice.

To more specifically isolate just APCC, we then introduced a dissecting microscope and scalpel to macrodissect APCC materials[24]. We prepared serial FFPE sections including CYP11B2 IHC and unstained slides, and macrodissected APCC regions by referring to CYP11B2 IHC slides. This method improved the precision compared to traditional macrodissection without microscopy, and is easier, faster and cheaper than laser-capture microdissection. We also improved the NGS method to limit false-positive findings from amplicon-specific errors, by the addition of a second aldosterone targeted sequencing panel (APAv2) that targets essentially the same genes as APAv1, but using different amplicons.

Hence, by making replicate NGS libraries on DNA from a single APCC sample (using both APAv1 and APAv2 panels), panel specific errors are excluded as they are not present in both replicates. This approach enables high confidence mutation detection even at relatively low APCC purity. These improvements support the feasibility/usefulness of IHC-guided NGS in revealing the underlying pathobiology in minute FFPE tissue samples.

Although it is currently still challenging, an expansion of the NGS analysis of DNA from APCC to whole exome or whole genome would help to explain the potential causes for CYP11B2 expression in the majority of APCC that do not have mutations in known aldosterone-driver genes (Figure 1).

7. Conclusions and perspectives

In this review, we described the current studies on APCC which have highlighted potential mechanisms for cellular and genetic development of PA. Since there is accumulating evidence that APCC overproduce aldosterone because of somatic gene mutations and represents a precursor for some APA, confirmation by *in vitro* and *in vivo* models is highly warranted. The evolving methods that make use of archival adrenal FFPE samples supports the feasibility and usefulness of IHC-guided NGS. As technology develops, whole exome sequencing or whole genome sequencing, as well as non-genomic analyses, may reveal new insights into development and role of APCC in PA.

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Figure 1. Somatic mutation prevalence in aldosterone-producing adenoma (APA) and aldosterone-producing cell clusters (APCC)

The distinct mutation pattern in APA (European cohorts) and APCC is shown. *KCNJ5* is the most commonly mutated gene in APA, whereas *CACNA1D* mutations are more common in APCC (22/84, 26%). *CTNNB1* mutations are also described in APA, while no APCC harbor them. APA chart was adapted from references 13 and 14, in which all four genes (*KCNJ5*, *CACNA1D*, *ATP1A1* and *ATP2B3*) were analyzed in independent cohorts. Note that *CTNNB1* was not examined in reference 13, therefore the prevalence is 10/198. APCC chart was adapted from references 19 and 21. Mutations in APCC include both reported and unreported ones in APA.

Figure 2. Histology and IHC of a representative aldosterone-producing cell cluster (APCC)
A representative APCC from a normotensive patient is shown in **A–D**. **A**. Hematoxylin and eosin (H&E) staining shows zona glomerulosa (ZG) and zona fasciculata (ZF) cells in APCC are not distinguishable from surrounding ZG and ZF cells, and adrenal zonation is preserved with no obvious cellular atypia or capsulization. **B**. CYP11B2 immunohistochemistry (IHC) shows distinct positive cluster of cells (APCC). **C–D**. APCC negative staining for CYP17A and CYP11B1. Adapted from **Supplemental** Figure 1 in reference 24.