Old and New Concepts in the Molecular Pathogenesis of Primary Aldosteronism

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Old and New Concepts in the Molecular Pathogenesis of Primary Aldosteronism

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Primary aldosteronism (PA) was first reported by Jerome Conn from the University of Michigan and is characterized by hypertension with excessive, autonomous production of
aldosterone relative to suppressed renin levels. Once thought to be a rare condition, it is now known to be the most common form of endocrine hypertension. The overall prevalence in the general hypertensive population was recently demonstrated to be 5.9% increasing with the severity of hypertension (3.9-11.8%, grade I-III hypertension) to as much as 20% in patients with resistant hypertension. An early diagnosis followed by correct treatment has an important impact on clinical outcome because it can reverse the increased risk of cardiovascular and cerebrovascular complications associated with PA.

PA can be familial caused by either of the 4 currently recognized subtypes (Familial Hyperaldosteronism types I-IV) or arise sporadically in which the aldosterone excess is unilateral [usually caused by an aldosterone-producing adenoma (APA)] or bilateral [usually caused by bilateral adrenal hyperplasia (BAH)]. The unilateral and bilateral forms of PA should be differentiated because a patient with unilateral APA is most appropriately treated by adrenalectomy, which normalizes or improves blood pressure in 84% of cases, and a patient with BAH is treated in almost all cases with a mineralocorticoid antagonist. Details for the diagnosis of PA and recommendations for the protocol and interpretation of adrenal venous sampling are described in detail elsewhere.

Until 2011, the genetic basis of PA was largely unknown when in an unprecedented development germline mutations causing new familial forms of PA and somatic mutations driving aldosterone excess in the majority of sporadic unilateral APAs were uncovered. The development of specific monoclonal antibodies to the highly homologous human CYP11B2 and CYP11B1 enzymes identified novel histological structures and evolved our understanding of the pathophysiology of aldosterone overproduction. Herein we comprehensively cover what is old and what is new in the rapidly developing field of the
pathogenesis of PA and focus on discoveries from genetics to histopathology of the last 3 years.

**Familial forms of hyperaldosteronism**

Familial hyperaldosteronism is estimated to account for less than 6% of all referred cases of PA.\(^\text{11}\) Familial hyperaldosteronism type I (FH type I, previously referred to as glucocorticoid-remediable aldosteronism) was the first genetically-defined form of human hypertension.\(^\text{12}\) The molecular basis of FH type II is unknown whereas the genetic determinants of FH types III and IV were identified with the advent of next-generation sequencing methods. The genetic basis and clinical phenotype of each of the known familial forms of PA are summarized in table 1.

**Familial hyperaldosteronism type I**

Familial hyperaldosteronism type I (FH type I) accounts for less than 1% of diagnosed cases of PA.\(^\text{11}\) This disorder displays a high degree of inter- and intra-familial phenotypic variability but is usually an early-onset form of hypertension associated with a high incidence of stroke. FH type I is caused by a chimaeric gene that comprises the promoter of the 11β-hydroxylase gene (CYP11B1) fused to the coding region of the aldosterone synthase gene (CYP11B2).\(^\text{12}\) Gene expression of the CYP11B1/CYP11B2 chimera is therefore regulated by adrenocorticotropic hormone, as for CYP11B1, and not by angiotensin II like CYP11B2. Unlike wild-type CYP11B2, the expression of aldosterone synthase from the chimaeric gene is suppressed by glucocorticoids, such as dexamethasone, because they suppress adrenocorticotropic hormone. CYP11B2 expression is normally restricted to the zona glomerulosa (zG) whereas patients with FH type I express the chimaeric gene, and the coding region of aldosterone synthase in the zona fasciculata (zF).\(^\text{12}\) Aldosterone synthase activity is
thus co-localized with cortisol that can be metabolized further by the 18-hydroxylase and 18-oxidase activities of aldosterone synthase to overproduce the hybrid steroids 18-hydroxycortisol and 18-oxocortisol as is characteristic of this condition.12

Familial hyperaldosteronism type II

The clinical and biochemical phenotype of FH type II is indistinguishable from sporadic PA11 and is not suppressible by glucocorticoids.13 FH type II is diagnosed when at least two family members present with PA who are negative for the chimeric gene which causes FH type I, and for FH type III and FH type IV germline mutations (described in more detail later). The prevalence of FH type II is difficult to evaluate considering the high prevalence of hypertension in the adult population14 and the prevalence of PA in the general population with hypertension3 and therefore the chance finding of 2 or more adult family members with a diagnosis of PA should be considered. Nonetheless, an estimated prevalence has been reported as 3.2-5.5% of all cases of PA.10,11,15 Although an association of FH type II with a genetic locus on chromosomal region 7p22 has been reported for some affected families,16-18 no mutations in candidate genes have been identified to date despite direct Sanger sequencing of multiple candidate genes and next generation sequencing of the linked locus and therefore the genetic basis of FH type II remains unknown.10

Familial hyperaldosteronism type III associated with KCNJ5 mutations

Familial hyperaldosteronism type III (FH type III) was first described by Geller et al.19 in 3 family members, the male index case and his 2 daughters, who presented with hyperaldosteronism and severe hypertension from childhood with hypokalemia and high levels of the hybrid steroids 18-oxocortisol and 18-hydroxycortisol.19 The index case originally came to medical attention as a 5 year old some 50 years previously; treatment
required bilateral adrenalectomy at 9 years of age which normalized blood pressure and the serum K⁺ concentration within 1 week of surgery. The daughters were treated similarly: the resected adrenals from all 3 patients were greatly enlarged and showed extensive hyperplasia of the zF.

The arrival of next-generation sequencing technology later revealed the presence of a germline heterozygous mutation in the KCNJ5 gene (encoding the G protein-activated inward rectifier potassium channel 4, GIRK4) that associated with the disorder in both father and daughters. The mutated K⁺ channel GIRK4 p.Thr158Ala) displayed a loss of selectivity for K⁺ and an increase in Na⁺ conductance that depolarizes the cell membrane. In zG cells, membrane depolarization causes the opening of voltage-dependent Ca²⁺ channels and Ca²⁺ influx which activates Ca²⁺ signaling pathways and leads to an increase in aldosterone production. Since this original case, other families with FH type III and patients with germline KCNJ5 mutations have been described with a clinical phenotype ranging from mild to severe (Table 1; Figure 1).

**Familial hyperaldosteronism type IV associated with CACNA1H mutations**

A new familial form of early-onset hyperaldosteronism has been described recently (Familial hyperaldosteronism type IV, FH type IV) and is caused by a germline mutation in the CACNA1H gene that encodes the T-type (low voltage activated) Ca²⁺ channel Ca₃.2 (Ca₃.2 p.Met1549Val). Gain-of-function mutations in CACNA1H have previously been associated with epilepsy, seizures and autism. Scholl et al. reported 5 unrelated individuals with early-onset PA with identical Met1549Val mutations. Genetic analyses of the patients’ families showed that 1 mutation occurred de novo and 4 occurred in the germline. In some kindreds, the Ca₃.2 p.Met1549Val variant segregated with the presentation of early-onset PA with severe hypertension; in another, the phenotype
displayed an incomplete penetrance with an intra-familial clinical presentation that varied from asymptomatic adults to the early-onset phenotype.\textsuperscript{24,27} This apparent incongruence with the autosomal dominant pattern of inheritance is presumably due to differences in age or to other genetic and environmental factors.\textsuperscript{27}

The physiology of the $\text{Ca}_\text{v}$3.2 p.Met1549Val channel was studied by patch clamp recordings of whole human embryonic kidney 293T cells expressing either the mutated channel or the wild-type $\text{Ca}_\text{v}$3.2 channel. $\text{Ca}_\text{v}$3.2 p.Met1549Val displayed a markedly slower channel inactivation and a shift of activation to less depolarizing potentials.\textsuperscript{24} The Met1549Val mutated form of $\text{Ca}_\text{v}$3.2 thus permits an increased $\text{Ca}^{2+}$ influx that in the steroidogenic cell lines NCI H295R and HAC 15, as for mutated GIRK4 channels that likewise cause an influx of $\text{Ca}^{2+}$, result in an increased expression of the $\text{CYP11B2}$ gene and aldosterone production.\textsuperscript{28}

\textit{A de novo} mutation affecting the same residue, $\text{Ca}_\text{v}$3.2 p.Met1549Ile, was identified shortly after in a study that reported 4 different germline $\text{CACNA1H}$ mutations.\textsuperscript{29} The Met1549Ile substitution was associated with early onset PA and multiplex developmental disorder (Table 1). The same study also reported $\text{Ca}_\text{v}$3.2 p.Ser196Leu and p.Pro2083Leu mutations associated with familial hyperaldosteronism and found a germline $\text{Ca}_\text{v}$3.2 p.Val1951Glu mutation in a patient with sporadic APA who was successfully treated by unilateral adrenalectomy.\textsuperscript{29}

**Germline CACNA1D mutations associated with primary aldosteronism with seizures and neurologic abnormalities (PASNA)**
In an attempt to identify new germline mutations associated with PA, candidate
genes with recurring somatic APA mutations were sequenced in 100 patients with early-onset PA. This approach identified 2 patients with de novo mutations in CACNA1D that encodes Ca\textsubscript{v}1.3, a subunit of an L-type (high-voltage activated) Ca\textsuperscript{2+} channel. The two different mutations- Ca\textsubscript{v}1.3 p.Gly403Asp and p.Ile770Met- affect amino acid residues of Ca\textsubscript{v}1.3 that are also substituted in sporadic APA (described later) with the latter identical to a somatic APA mutation. Unlike the somatic variants, the germline mutations cause an
extreme phenotype, with early onset severe hypertension and hyperaldosteronism and a
diagnosis in both patients of neurologic abnormalities that include cerebral palsy and
seizures referred to as PASNA.

have also been identified in patients with autism spectrum disorder. These patients
additionally presented with epilepsy and had a clinical phenotype resembling that of
patients with PASNA. Characterization of the electrophysiological effects incurred by the
mutations was demonstrated by patch clamp recordings in vitro. All 3 mutations confer a
gain-of-function phenotype with a predicted increase in Ca\textsuperscript{2+} inward currents which
would be expected to result in elevated plasma aldosterone concentrations in these patients
but regrettably, these data are unavailable.

Germline KCNJ5 mutations associated with sporadic primary aldosteronism

Germline KCNJ5 mutations were identified in 3 of 251 patients with a florid form of sporadic
BAH. The mutations (p.Arg52His, p.Glu246Lys and p.Gly247Arg) were located at some
distance from the selectivity filter of the GIRK4 K\textsuperscript{+} channel. Despite this, the Arg52His and
Glu246Lys mutations caused cell depolarization and increased aldosterone production in
vitro in contrast to the Gly247Arg mutation that had no functional effect. A rare	nonsynonymous single-nucleotide polymorphism rs7102584 was also identified in 12 of 251
white Australasian patients (5%) with PA compared with 22 of 1075 individuals (2%) in the
international 1000 genomes cohort. In the 12 patients with PA carrying the rs7102584
(p.Glu282Gln) polymorphism, 9 were diagnosed with BAH and 3 with APA.33

Sporadic forms of hyperaldosteronism caused by somatic mutations

Over half of APAs carry somatic gain-of-function mutations in genes encoding ion
channels or transporters (KCNJ5, CACNA1D, ATP1A1, ATP2B3) that can account for excessive
production of aldosterone. Somatic mutations have also been described in APA that are
found in cortisol-producing adenomas (CTNNB1, PRKACA and GNAS) although PRKACA and
GNAS mutations are rare in APA. The somatic APA mutations reported to date are listed in
Table S1.

Somatic KCNJ5 mutations in APA

The original somatic APA mutations described by Choi21 in the KCNJ5 gene cause
GIRK4 p.Gly151Arg and p.Leu168Arg substitutions in or near the pore of the channel,
respectively. Both mutations, as for others described subsequently, disturb the selectivity
properties of the channel as for the p.Thr158Ala mutation associated with FH type III
described above. At least 17 somatic APA KCNJ5 mutations have been described to date (Fig.
1, Table S1), but the p.Gly151Arg and p.Leu168Arg substitutions predominate accounting for
all 34% of APA with GIRK4 mutations of 380 APA recruited through the European Network
for the Study of Adrenal Tumours34 (ENS@T, http://www.ensat.org) and 47% of APA in an
international series of 351 APA.35 Somatic KCNJ5 mutations in APA are more frequent in
women than in men and compared to non-carriers, are associated with a more pronounced hyperaldosteronism, a younger age and larger tumor size.\textsuperscript{34,36}

The overall prevalence of APA with \textit{KCNJ5} mutations was determined in a meta-analysis of 1636 patients as 43\% (range 12-80\%) with a higher reported frequency in Japan and China compared to European countries.\textsuperscript{36} Because patients with APA carrying a \textit{KCNJ5} mutation have higher concentrations of the aforementioned hybrid steroid 18-oxocortisol in peripheral blood,\textsuperscript{37} the reported predominance of APA with a \textit{KCNJ5} mutation in patients from Japan explains the utility of using 18-oxocortisol in peripheral plasma as a potential biomarker to differentiate patients with APA from those with BAH\textsuperscript{38} whereas this is not useful in patients from Europe.\textsuperscript{39} However, the measurement of a panel of 7 steroids, that includes the hybrid steroids 18-oxocortisol and 18-hydroxycortisol, in peripheral plasma by liquid chromatography-tandem mass spectrometry correctly classified 92\% of 79 patients with APA according to genotype with a correct call for 26 of 27 patients with APA with a \textit{KCNJ5} mutation.\textsuperscript{37}

In a recent development, macrolide antibiotics were identified as selective inhibitors of GIRK4 p.Gly151Arg and p.Leu168Arg compared with the wild-type GIRK4 channel and the macrolide compound roxithromycin ablated \textit{CYP11B2} gene expression and inhibited aldosterone production induced by the expression of either GIRK4 p.Gly151Arg or p.Leu168Arg in steroidogenic cell lines.\textsuperscript{40} Macrolides have a potential use in the diagnosis of patients with an APA carrying a mutated GIRK4 channel by measuring drug-induced decreases in aldosterone concentrations- possibly in tandem with peripheral venous steroid profiling- and in their treatment.

\textbf{Somatic \textit{ATP1A1} and \textit{ATP2B3} mutations in APA}
Shortly following the discovery of \textit{KCNJ5} mutations, somatic APA mutations were described in the \textit{ATP1A1} and \textit{ATP2B3} genes, encoding Na\(^+\)/K\(^+\)-ATPase 1 and Ca\(^{2+}\)-ATPase 3, respectively,\(^{41,42}\) that combined account for 7\% of APA in patients of European ancestry and can result in an increase in \textit{CYP11B2} gene expression and aldosterone production\(^{43,44}\) (Table S1 lists the \textit{ATP1A1}/\textit{ATP2B3} mutations described to date). APA mutations in ion transporters and channels were thought to share a common mechanism of activation of Ca\(^{2+}\) signaling, but two mutations in the Na\(^+\)/K\(^+\)-ATPase, p.Leu104Arg and p.Val332Gly, reportedly result in intracellular acidification, with no detectable increase in intracellular Ca\(^{2+}\) concentration, that causes the overproduction of aldosterone.\(^{45}\) Germline mutations in ATPases have not been described to date presumably because they are incompatible with life.

**Somatic \textit{CACNA1D} mutations in APA**

Somatic APA mutations in \textit{CACNA1D} that cause substitutions at conserved sites of Ca\(_{\text{v}1.3}\) were first described in two reports.\(^{30,41}\) The mutations determine an impairment of the ion channel gating mechanism such that both activation and inactivation of the channel are affected and an increase in Ca\(^{2+}\) influx results.\(^{30,41}\) The number of different somatic APA mutations in \textit{CACNA1D} are numerous with at least 31 substitution mutations of 25 different amino acid residues reported (Table S1). Somatic \textit{CACNA1D} mutations are associated with APA of a smaller size\(^{41,44}\) and, with a reported prevalence of 9.3\%, comprise the second largest group of APA after those with a \textit{KCNJ5} mutation.\(^{44}\) A highly selective antagonist of Ca\(_{\text{v}1.3}\), 1-(3-chlorophenethyl)-3-cyclopentylpyrimidine-2,4,6-(1H,3H,5H)-trione has been developed as a potential novel therapeutic approach for Parkinson’s disease, displaying 600-times selectivity over Ca\(_{\text{v}1.2}\)\(^{46}\) and selective antagonism of Ca\(_{\text{v}1.3}\) may be a target for the treatment of aldosterone overproduction in patients with PA.\(^{47}\)

**Somatic \textit{CTNNB1} mutations in APA**
The canonical Wnt (Wnt/β-catenin) pathway controls key developmental gene expression programs and constitutive activation of this pathway is involved in the pathogenesis of many human cancers. In the absence of extracellular Wnt ligands, glycogen synthase kinase 3β, as part of the multi-protein Axin complex, phosphorylates specific serine and threonine residues in the N-terminal region of β-catenin which is then degraded via the ubiquitin-proteosome pathway. Binding to the Frizzled/LRP (lipoprotein receptor-related protein) receptor inhibits the Axin complex and β-catenin is stabilized, accumulates and translocates to the nucleus to activate LEF/TCF (T-cell factor/lymphoid enhancer factor) transcription factors thereby activating Wnt target gene expression programs.

Activating somatic mutations in the CTNNB1 gene, that encodes β-catenin, have been reported at a similar prevalence in both adrenocortical adenomas (27% of 26 tumors, with a higher proportion in non-functioning adenomas compared with cortisol-producing adenomas) and adrenocortical carcinomas (31% of 13 tumors). Most mutations affected a phosphorylation site (Ser45) for glycogen synthase kinase 3β.

A high proportion of APAs (70%) display constitutive activation of Wnt/β-catenin signaling that is caused, at least partly, by a decrease in the expression of SFRP2 (Secreted Frizzled Related Protein 2) an endogenous inhibitor of Wnt/β-catenin signaling. Somatic mutations in the CTNNB1 gene have been identified in 2-5% of APA. All mutations target exon 3 and almost all affect serine and threonine residues at the β-catenin N-terminus resulting in an accumulation of β-catenin. Therefore, β-catenin mutations are present in APA as in other adrenocortical tumors where they play a role in adrenocortical tumorigenesis.

Somatic APA CTNNB1 mutations have also been described in 2 pregnant women and a woman post-menopause with highly up-regulated adrenal expression of luteinizing hormone/choriogonadotropin receptor and gonadotropin releasing hormone receptor.
leading to the suggestion that pregnancy could trigger the overexpression of these gonadotropin receptors via a mechanism mediated by constitutive activation of β-catenin.\textsuperscript{53}

**Histopathology of aldosterone production**

The development of specific monoclonal antibodies against the key steroidogenesis enzymes CYP11B2 and CYP11B1\textsuperscript{54} has transformed the interpretation of the histopathology of PA and identified a new histological structure called the aldosterone-producing cell cluster (APCC).\textsuperscript{55-58} APCCs are distinct clusters of zG cells identified by strong CYP11B2 immunostaining and an absence of CYP17 staining and are located in the subcapsular portion of the adrenal cortex that penetrates into the cortisol-producing cells of the zF.\textsuperscript{54,59} At least 1 APCC was identified in 69% of an autopsy series of 127 normal adrenals\textsuperscript{59} and 50% of 22 adrenals had an APCC in the tissue adjacent to an APA.\textsuperscript{60} The physiology of aldosterone production changes with age with a transition from continuous expression of CYP11B2 in the zG in the young to a discontinuous expression with an increased expression of APCCs in older subjects.\textsuperscript{59} The adrenals of children aged below 12 years show a conventional zonation of the adrenal cortex without APCCs\textsuperscript{61} with the earliest documented case of an APCC occurring in a 12 year old male.\textsuperscript{59} Transcriptome analysis of APCC and the 3 zones of the adrenal cortex demonstrated the highest expression of CYP11B2 in APCCs and the similarity of the APCC and the zG transcriptomes.\textsuperscript{62}

A high proportion of APCCs in the normal adrenal glands from kidney donors carry mutations in genes that are mutated in APAs (35% of 23 APCCs have mutations in CACNA1D or ATP1A1) leading to the proposal that APCCs are precursors of APAs.\textsuperscript{62} Although APCC with KCNJ5 mutations, the most frequently recurring mutation in APAs, were not identified, a histological structure with a transitional phenotype from APCC (subcapsular region) to microAPA (outer region), called APCC-to-APA transitional lesions, have been described in
which KCNJ5 mutations were identified in the microAPA portion of the larger lesions (>3mm). This supports the hypothesis of the APCC to microAPA transition and suggests that KCNJ5 mutations could occur as a second genetic hit.61,63 The number of the reported transitional lesions are however few with 6 known cases to date.

If present adjacent to an APA, the production of aldosterone from APCCs is presumably biochemically autonomous given the suppressed renin-angiotensin-system, but their contribution to aldosterone excess is likely to be negligible, since the APA is probably the main source of aldosterone production. However, in the normal adrenal cortex of elderly persons APCCs may be a driver of hypertension and contribute substantially to age-related cardiovascular risk.59

APA display different patterns of CYP11B2 and CYP11B1 immunostaining that can be homogeneous and dominant for CYP11B2, heterogeneous for CYP11B2 and interspersed with patches of CYP11B1, or CYP11B2-negative.56,60 CYP11B2 immunoreactivity is significantly higher in microadenomas than in macroadenomas and patients with the smaller tumors display disproportionately higher levels of aldosterone production64 and CYP11B1 immunostaining is associated with glucocorticoid overproduction in some APA.65

The adrenal tissue adjacent to an APA can display micronodules or macronodules that can carry a somatic mutation of a different genotype to the APA.58,66,67 In some infrequent cases, a secondary nodule is the source of the excess aldosterone production and in these circumstances the adenoma is usually CYP11B2-negative.56,68

Conclusion

Advances in sequencing technologies and immunohistochemistry have advanced our understanding of the pathogenesis of PA and shown that the pathological production of
aldosterone in this most common form of endocrine hypertension is not as straightforward as classically contended.

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**Disclosures**

The authors having nothing to disclose

**References**


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<th><strong>PA subtype</strong></th>
<th><strong>Genetic variant</strong></th>
<th><strong>Affected protein</strong></th>
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<th><strong>Clinical phenotype</strong></th>
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**Table 1. Germline mutations associated with primary aldosteronism**

FH, Familial hyperaldosteronism; PA, primary aldosteronism; APA, aldosterone-producing adenoma; BAH, bilateral adrenal hyperplasia; PASNA, PA with seizures and neurologic abnormalities; *CYP11B1*, gene encoding 11β-hydroxylase; *CYP11B2*, gene encoding aldosterone synthase; ACTH, adrenocorticotropic hormone; zF, *zona fasciculata*; GIRK4, G protein coupled inwardly rectifying potassium channel 4;
Ca$_v$3.2, Ca$^{2+}$ channel, voltage-dependent, T type, $\alpha$ 1H subunit; Ca$_v$1.3, Ca$^{2+}$ channel, voltage-dependent, L type, $\alpha$ 1D subunit; DEX, dexamethasone; ADX, adrenalectomy; MRA, mineralocorticoid receptor antagonist; CCB, Ca$^{2+}$ channel blocker. *The hybrid steroids are 18-hydroxycortisol and 18-oxocortisol; †de novo mutation also identified, ‡de novo mutation; #rare nonsynonymous single-nucleotide polymorphism rs7102584 present in 12 of 251 patients (5%) with PA compared with 22 of 1075 individuals (2%) in the international 1000 genomes cohort. In the 12 patients with PA carrying the rs7102584 (Glu282Gln) polymorphism, 9 were diagnosed with BAH and 3 with APA.33
Figure 1. Germline and somatic mutations in GIRK4 associated with different forms of primary aldosteronism

Different somatic and germline mutations are indicated in GIRK4 encoded by the KCNJ5 gene.
ONLINE SUPPLEMENT

Old and New Concepts in the Molecular Pathogenesis of Primary Aldosteronism

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Table S1. Somatic mutations associated with aldosterone-producing adenomas

GIRK4, G protein coupled inwardly rectifying potassium channel 4; Ca, 1.3, Ca^{2+} channel, voltage-dependent, L type, α1D subunit; Ca, 3.2, Ca^{2+} channel, voltage-dependent, T type, α1H subunit

*To our knowledge, the above table shows all somatic mutations in aldosterone-producing adenomas reported to date.

†Most frequently occurring mutations in aldosterone-producing adenomas

‡Mutations also present in adenomas co-secreting cortisol